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Molecular characterization and RFLP profile of an *Inocybe* (Inocybaceae, Agaricales) species isolated from Tlaxcala (Mexico): evidence for a new species in the subgenus *Mallocybe*

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With 4 figures and 3 tables

Abstract: *Inocybe* species are ectomycorrhizal fungi that have the potential to serve as inoculants for forestry plant species, especially in soils found in extreme conditions. Therefore, it is important to know its diversity, so the best adapted species for use in revegetation programs could be selected. The present research focused on the molecular characterization of one fungal morphotype in the *Inocybe* subgenus *Mallocybe*, a major fungal component in a pine plantation established on a disturbed area in the state of Tlaxcala, Mexico, as well as to generate a valid molecular marker for early and fast detection in field studies. The Internal Transcribed Spacer of the ribosomal DNA was amplified, sequenced and digested with four restriction enzymes in order to detect restriction fragment length polymorphisms (RFLPs). The sequence data of the product obtained has 807 base pairs (bp) and its BLAST analysis in the GenBank and UNITE databases resulted in an 84% similarity to *Inocybe dulcamara* f. *pygmaea*. The phylogenetic analysis indicates that the closest related species to the Mexican species is *Inocybe terrigena*. The RFLPs differ in important ways from the sequences of other species of *Inocybe* subgenus *Mallocybe*, and this supports their importance as tool for identification of the species in field samples. The evidence obtained in this research suggests that the Mexican species is different from other species in the subgenus *Mallocybe* previously studied since a molecular and phylogenetically point of views.

Key words: Ectomycorrhizal fungi, Fingerprinting, ITS, UNITE, Phylogenetic analysis.

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Introduction

Inocybe belongs to the family Inocybaceae, which includes near 700 species worldwide (Matheny et al. 2009). This genus forms ectomycorrhizal symbiosis with Gymnosperms and Angiosperms, especially in the early stages of the ectomycorrhiza formation (Kuyper 1986, Bowen 1994, Matheny 2003). *Inocybe* is one of the most common genus in young forests (Reverchón et al. 2010), contributing to its biodiversity and resilience (Kranabetter et al. 2009). Nevertheless, the biotechnological utilization of *Inocybe* species represents a challenge for scientists because basidiospores do not germinate under laboratory conditions and, frequently, they are not successfully cultured on synthetic media (Boidin 1986). In spite of this, they have an enormous potential for being used in forestry programs for the rehabilitation of disturbed areas affected by either biotic or abiotic factors (Cullings 2001, Barroetaveña 2007). Species of *Inocybe* are especially useful as they are predominant fungi in acidic or saline soils (Ishida 2009), as well as being able to survive under extreme high or low temperatures (Cullings 2001, Jumpponen 2002, Barroetaveña 2007, Gao 2010). But these capabilities could depend on specific fungal species or even individuals. Thus, the characterization or identification of *Inocybe* species is an important element to consider for further biotechnological management (Samson 1986).

Fungal species from the American continent have usually been identified based on European descriptions and monographs and, until now, there has been no taxonomic treatment for *Inocybe* and its allies in North America, with the exception of the work of Cripps et al. (2010) for the Rocky Mountains alpine species in the subgenus *Mallocybe*. Furthermore, according to Liang et al. (2007) the mycobiota of the Neotropical forests, including Mexican territories, has been poorly studied, and being the European and North American ectomycorrhizal fungi (ECMF) the most studied, the exploration of new territories could lead to the discovery of, i) species of *Inocybe* different from those places, which could correspond to new taxa (Cripps et al. 2010, Reverchón et al. 2012), ii) cryptic species hidden for very few morphological variations.

In addition, Matheny (2009) pointed out that a significant number of insufficiently known species exists in herbaria (as specimens) and in the GenBank database (as DNA sequences).

Matheny (2005) did a phylogenetic analysis of *Inocybe* using RPB1, RPB2 and LSU, and obtained five clades, one of them called the *Mallocybe* clade, which included *Inocybe dulcamara* (Pers.) P.Kumm. and allies. Kuyper (1986) proposed *Mallocybe* as a subgenus of *Inocybe*.

Inter Genetics Spacers (IGS) and Internal Transcripts Spacers (ITS) are polymorphic and variables regions useful in phylogenetic and taxonomic studies because they are highly repeated genetic regions of ribosomal DNA (rDNA), in specific chromosomes that include well preserved 18S, 5.8S and 28S genes (Henrion et al. 1994). In particular, the ITS region is considered as a good element with high resolution at specific levels for many fungal taxa (Nilsson et al. 2008, Ryberg et al. 2008, Seifert 2009, Dentinger et al. 2010, Schoch et al. 2012). Simultaneous use of the sequencing and fingerprinting techniques of informative regions for the analysis, along with molecular identification,

allows a more precise characterization of ECMF and, consequently, a better resolution at the specific level (Gardes & Bruns 1996, Walbert et al. 2010). Additionally, the molecular markers can constitute a good tool for detection of the species, when it is used in field experiments, without considering necessary the obtaining of the DNA sequence.

The aim of this work was to define the identity of one species placed in the *Inocybe dulcamara* complex with some variations on its typical morphological characters, collected from a deforested area in Tlaxcala (Mexico), applying molecular tools such as ITS sequencing and phylogenetic analysis as well as fingerprinting PCR-RFLP which could be useful as a tool for practical identification in field samples.

Materials and methods

Fungal material: The study was based on basidiomes morphologically identified as *Inocybe dulcamara* by Kong Luz A., Herbarium TLXM, using specialized keys and descriptions (Smith et al. 1979, Phillips 1983, 1991, Bon 1988, Metzler & Metzler 1992, Kobayashi 2002, 2003, 2005) and the Munsell color key (U.S. Department of Agriculture 1975). The specimen was collected from a plantation of *Pinus cembroides* Zucc. at Cerro Tepecticpac, located in the state of Tlaxcala. This species was one of the major components either of the pine-oak residual forest or from the introduced pine plantations in this place. It was found four years after the introduction of the pine plantation, from 1993 to 1998, in soils with alkaline pH, sandy texture, high content of calcium and low levels of available phosphorus. For these reasons, this species could be a good candidate for being used in revegetation programs (Varela-Fregoso 2000).

The morphological characters of the specimen studied are: Pileus 25–45 mm in diameter, dry, hemispheric, sometimes with a shallow central umbo, dull yellowish brown (10YR3/6), with a radially fibrilous-rimose surface; margin smooth, entire. Lamellae adnate, wide to ventricose, margin smooth, dark reddish brown (10YR5/4). Cortina fugacious, formed by fibrils remain at pileus margin and on stipe. Stipe 28–60 mm long × 2.5–4 mm wide, straight to sinuate, hollow, yellowish brown (10YR3/4), surface fibrous, with basal tomentus. Spores olive yellow, 7.5–10.5 × 5–5.5 µm, smooth, phaseoliform, with apex obtuse and lighter. Cheilocystidia clavate to pyriform, 9–13 × 70–85 µm, pale, thin-walled (Fig. 1A–C). Darker color of basidiomes, the absence of olive tint on lamellae, the slightly small yellow olive spores, and the longer cheilocystidia distinguish the Mexican specimen from the description of *Inocybe dulcamara* given by Cripps et al. (2010) from North America. A voucher specimen was deposited at the TLXM Herbarium (reference number GF2321), Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala.

DNA extraction from Basidiomes: Samples of the GF2321 specimen were selected, cleaned and macerated with liquid nitrogen. Then the macerated fungus was set in 1.5 ml Eppendorf tubes and preserved to -20°C. The extraction of DNA was performed with a DNAzol reagent as indicated by the manufacturer, and purified and preserved at -20°C.

Amplification of ITS region with PCR: Primers for ITS amplification of rDNA (with sequences of ITS1/5.8S/ITS2) were selected in accordance to White et al. (1990), Gardes & Bruns (1991, 1993), and Lian et al. (2003), named ITS1-F and ITS4 and then synthetized (Uniparts, S.A. de C.V., Distrito Federal, Mexico). Components for the reactions were: 10 µl of DNA total (10 ng), 5 µl of 10 × Taq Buffer with KCl (100mM Tris-HCl, 500 mM KCl, nonidet P40), 4 µl of $MgCl_2$ 25 mM, 1 µl of dNTP´s 10 mM, 1 µl of the primer ITS1F 20 mM, 1 µl of the primer ITS4 20 mM, 27.5 µl of bidistilled water, and 2.5 units of Taq Polymerase (Fermentas®). The PCR amplification was performed in a thermocycler, My Cycler (Bio-Rad®), with 30 cycles comprising the following stages: initial denaturalization at 94°C for 5 min, 30 cycles for denaturalization at 94°C lasting one min, annealing temperature at 45°C for one min, and polymerization at 72°C for one min, finishing with 7 min of polymerization at 72°C. Controls without DNA were run to assure there was no contamination in

Fig. 1. Mexican specimens from *Inocybe dulcamara* complex. A. Basidiomes, bar = 10 mm. B. Cheilocystides, bar = 50 µm. C. Spores, bar = 10 µm.

the reagents. The amplifications obtained were confirmed and analyzed by electrophoresis in 1.0% of agarose gel with ethidium bromide (0.5 µg/l) during a 15 min period (Sambrook et al. 2001).

Molecular sizes of bands were determined utilizing a Gene Ruler™ 100 pb DNA Ladder (Fermentas®), the bands were then made visible with a UV light under diffused light conditions (Ultra•Lūm®) and finally photographed with a Gel Doc XRTM 170-8170 (BioRad®). Amplicons were purified with the Wizard SV Gel Kit and PCR Clean-Up System (Promega®) as indicated by the manufacturer. The amplicon sequencing was performed in an automatic sequencer, ABI-Prism 3100 (Applied Biosystems®), at the Divisional Laboratory of the Molecular Biology, Universidad Autónoma Metropolitana-Iztapalapa.

To determine the sequence similitude and its possible identification, the nucleotide sequences of the studied species was compared with sequences included at the databases GenBank and UNITE using the software Basic Local Alignment Search Tool (BLAST). The sequence was deposited in GenBank.

Phylogenetic analysis: To determine the phylogenetic position of the target sample, sequences data of the ITS1/5.8S/ITS2 regions from the study by Cripps et al. (2010) were used. Additionally, two sequences of *I. terrigena* not considered in the study of Cripps et al. (2010) were also included. The sequences used as close outgroups for phylogenetical reconstruction were taken from Cripps et al. (2010). In total, 34 sequences data were obtained from the GenBank database (Table 1).

The sequences were aligned using Clustal X v.2.1 in a complete mode (Larkin et al. 2007) and the data matrix was edited in Mesquite v.304 for Windows (Maddison & Maddison 1997–2010). Phylogenetic analysis was performed with a posterior probabilities algorithm from the MrBayes software for Windows considered as the most recommendable for the analysis of molecular data (Ronquist & Huelsenbeck 2003), with the option lset nst=6rates=invgamma, with mcmcp ngen=6000000, and the consensus tree was edited with the FigTree v.1.4.2 program.

Fingerprinting PCR-RFLP generation: The amplified products were processed by digestion with the restriction enzymes *Hae*III, *Eco*RI, *Alu*I and *Pst*I, at 37°C for 16 h, using 10 ng of amplification products of ITS region with two units of each enzyme, a 2 µl enzyme buffer for each enzyme (supplied by the manufacturers) and a 16 µl deionized, sterile H_2O per sample. Results were confirmed and analyzed by electrophoresis in agarose gels (2.5%), and the gels stained with ethidium bromide (0.5 µg/l) for 15 min (Sambrook et al. 2001), including the molecular marker (Gene Ruler™ 100 pb DNA Ladder, Fermentas®). The analysis of bands was performed and photographed with the Quantity One software and the Gel Doc XRTM 170-8170 (BioRad®).

Results

Molecular identification of the Mexican *Inocybe:* The amplified ITS region of the rDNA from the basidiome of *Inocybe* TLXM (GF2321) resulted in one amplicon of 828 pb. The sequence consisted of 807 nucleotides (accession number in GenBank KR817255) and had an 84% similarity to that obtained at the GenBank as *Inocybe dulcamara* f. *pygmaea* (accession number GU980628.1). This result agrees with that obtained using the UNITE database. This percentage of similarity is too low for considering this sample as belonging to this taxon.

All the clades recovered in the phylogenetic analysis are the same found in the study of Cripps et al. (2010). The target sequence of this study is related with 63% of probability with the clade which includes three sequences of *I. terrigena* from Sweden, the United States and Italy (GenBank accession numbers: AM882864, GU980648 and JF908091, respectively). This last clade had a support of 92% (Fig. 2). The Mexican species is not phylogenetically related to *Inocybe dulcamara* neither to *Inocybe dulcamara* f. *pygmea*, as was previously suggested by the morphological identification or the blast analysis, respectively.

Genetic fingerprinting PCR-RFLP: Figure 3 shows the band patterns due to the restriction enzymes *Alu*I, *Eco*RI, *Hae*III, and *Pst*I. For *Alu*I, *Eco*RI and *Pst*I, the sum of the obtained band sizes is equal to the size of the complete sequence. Nevertheless, for *Hae*III, we obtained only two bands that do not add up to the same size as the original amplicon (Fig. 3, line 4).

This means that there are sites of recognition for this enzyme, but two of the fragments may be of similar size and it may be not possible to see them in the gel. We verified this with a bioinformatics analysis using the software SeqBuilder (DNASTAR®). The theoretical digestion of ITS sequences with *Hae*III was performed considering the

Table 1. List of GenBank sequences used in phylogenetic analysis.

No. Access Gene Bank	Specie	Voucher	Observations
FJ904178	*Crepidotus calolepis* (Fr.) P.Karst.	EL14-04	Sweden
AM882996	*Crepidotus mollis* (Schaeff.) Staude	EL4504	Sweden
GU980653	*Inocybe arthrocystis* Kühner	CLC1141	Independence Pass, Co, USA
GU980651	*Inocybe arthrocystis* Kühner	CLC1688	San Juans, Stony Pass, Co, USA
GU980654	*Inocybe arthrocystis* Kühner	SJ06-011	Sweden
GU980647	*Inocybe* cf *dulcamara* (Pers.) P.Kumm.	CLC1408	San Juans, Cinnamon Pass, Co, USA
GU980639	*Inocybe* cf *dulcamara* (Pers.) P.Kumm.	CLC1131	Beartooth Pass, Mt, USA
GU980628	*Inocybe dulcamara* f. *pygmaea* J.Favre	EL48-05	Norway
KR817255	*Inocybe* sp	GF2321	Tlaxcala, Mexico
GU980641	*Inocybe* cf. *dulcamara* (Pers.) P.Kumm	CLC1364	Independence Pass, Co. USA
GU980642	*Inocybe* cf. *dulcamara* (Pers.) P.Kumm	CLC1160	Beartooth Pass, Mt, USA
GU980645	*Inocybe* cf. *dulcamara* (Pers.) P.Kumm	EL33-05	Norway
GU980636	*Inocybe* cf. *dulcamara* (Pers.) P.Kumm	CLC1295	Loveland Pass, Co. USA
GU980643	*Inocybe* cf. *dulcamara* (Pers.) P.Kumm	EL59-05	Norway
GU980629	*Inocybe dulcamara* f. *pygmaea* J.Favre	J.Favre76bisType	Switzerland
FN550940	*Inocybe fuscomarginata* Kühner	EL10906	Sweden
GU980656	*Inocybe fuscomarginata* Kühner	BJ890718	Sweden
GU980657	*Inocybe fuscomarginata* Kühner	EL77-07	Sweden
GU980619	*Inocybe leucoloma* Kühner	CLC1431	San Juans, Emma Lake, Co, USA
GU980626	*Inocybe cf leucoloma* Kühner	EL1-06	Norway
GU980614	*Inocybe leucoloma* Kühner	Kuhner63-36Type	France
HM209795	*Inocybe squarrosoannulata* Kühner	K63-236Type	France
GU980609	*Inocybe squarrosoannulata* Kühner	SJ84030	Sweden
GU980612	*Inocybe* cf *squarrosoannulata* Kühner	CLC1490	Loveland Pass, Co, USA
GU980605	*Inocybe* cf *squarrosoannulata* Kühner	CLC1375	Independence Pass, Co, USA
GU980607	*Inocybe* cf *squarrosoannulata* Kühner	EL120-08	Beartooth Pass, Wy, USA
GU980601	*Inocybe substraminipes* Kühner	Kuhner70-48Type	France
GU980602	*Inocybe substraminipes* Kühner	CLC1649	Cumberland Pass, Co, USA
JF908091	*Inocybe terrigena* (Fr.) Kühner	97	Italy
AM882864	*Inocybe terrigena* (Fr.) Kühner	EL11704	Sweden
HQ604783	*Inocybe terrigena* (Fr.) Kühner	UBC:F19404	France
GU980648	*Inocybe terrigena* (Fr.) Kühner	EL 24-08	Beartooth Plateau, Wy, USA
FJ904122	*Mallocybe dulcamara*	EL 89-06	Sweden
GU980633	*Mallocybe leucoblema*	TAA128324	Estonia
GU980632	*Mallocybe leucoblema*	CLC1721	Cottonwood Pass, Co, USA
FN550936	*Mallocybe leucoblema*	EL6608	Sweden

Fig. 2. Consensus tree of 50% majority. The numbers on the branches are percentage of posterior probability. The clades named at right side correspond to species proposed by Cripps et al. (2010).

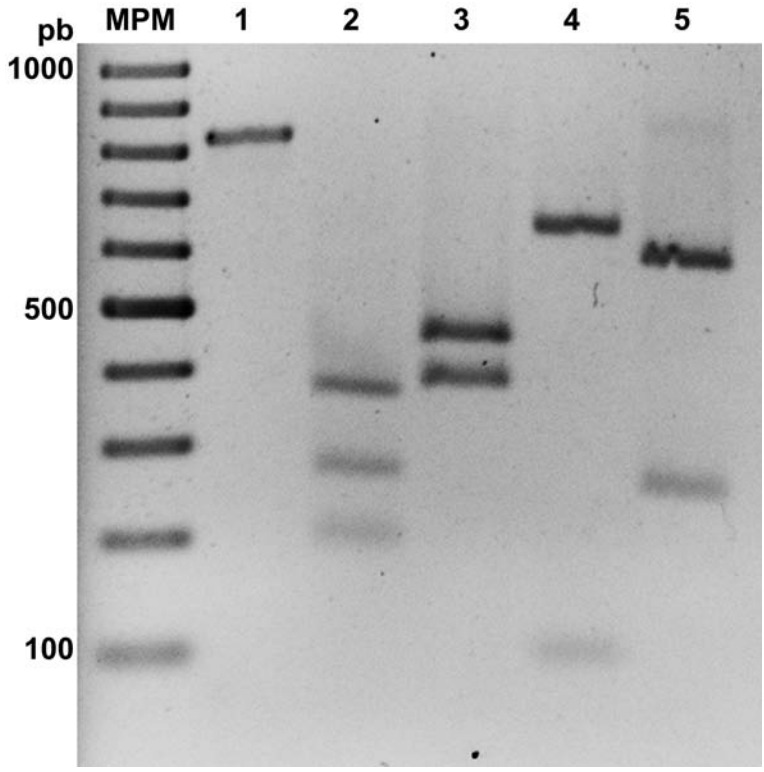

Fig. 3. Profile band of RFLP of the ITS region of the ribosomal DNA of *Inocybe dulcamara*, line 1: Amplicon with primers ITS1F and ITS4, line 2: Digestion fragment with *Alu*I, line 3: Digestion fragment with *Eco*RI, line 4: Digestion fragment with *Hae*III and line 5: Digestion fragment with *Pst* I.

807 bp of the target sequence. This analysis resulted in three fragments, two of them with very similar sizes (100 and 108 bp), which were impossible to detect in the gel (Fig. 4).

Based on this theoretical digestion, the sum of the sizes of the fragment represents the total size of the amplicon. Theoretical digestions with the other enzymes (*Alu*I, *Eco*RI and *Pst*I) resulted in fragment sizes similar to those observed in the gel (Table 2).

The simulated digestions through bioinformatics analysis with the enzymes used in this research allowed the determination of theoretical sizes of RFLP for the aligned ITS sequences of all the other species in the subgenus *Mallocybe* (Table 3), all of which showed differences from the Mexican species. This confirms that our target species is different from all the North American and European species included in this current research, including those with the most similar sequences, and that RFLP band patterns could be a useful tool for differentiating this species under practical conditions.

Fig. 4. Theoretical estimation of the digestion fragments of ITS of *Inocybe dulcamara* with restriction enzymes *Alu*I, *Eco*RI, *Hae*III and *Pst*I with SeqBuilder software.

Table 2. List of sizes obtained RFLP.

Restriction Enzyme	Number of fragments	Fragment size (pb)
*Alu*I	3	370, 254, 204
*Eco*RI	2	450, 392
*Hae*III	3	620, 100, 108
*Pst*I	2	569, 259

Discussion

Organisms considered to be the same species may vary depending on their geographical origins (Ryberg et al. 2010). Furthermore, many organisms with similar morphological characteristics are not necessarily the same species, and others that are morphologically different may belong to the same species (Petersen & Hughes 1999). This situation reflects the need for further taxonomic research efforts to characterize morphologically and molecularly fungal species from different world regions.

In the case of fungi, it is common to find species complexes due to the existence of cryptic species, which do not have sufficient morphological characters to make a proper identification, making it necessary to apply other type of techniques for bypassing the difficulties encountered during the taxonomical identification process, especially in species used in practical applications, such as the ECMF (Sato et al. 2007, Sheedy et

Table 3. Theoretical size of RFLP species *Inocybe* obtained by the SeqBuilder (DNASTAR®) program.

Species	Sequence size after alignments (pb)	*Alu*I (bp)	*Eco*RI (bp)	*Hae*III (bp)	*Pst*I (bp)
KR817255 Inocybe TLXM [a]	735	163, 370, 202	344, 391	529, 99, 107	478, 257
AM882864 Inocybe terrigena[c]	665	n/s	316, 349	n/s	n/s
GU980628 Inocybe dulcamara fma. pygmaea[bc]	691	132, 355, 204	311, 380	n/s	n/s
GU980639 Inocybe dulcamara[bc]	679	n/s	320, 359	n/s	n/s
GU980647 Inocybe dulcamara[bc]	644	n/s	303,341	n/s	n/s
GU980633 Inocybe leucoblema[c]	700	147, 553	325, 375	n/s	n/s
GU980642 Inocybe dulcamara[bc]	679	n/s	320, 359	n/s	n/s
GU980657 Inocybe fuscomarginata[c]	601	431, 170	281, 320	n/s	n/s
GU980629 Inocybe dulcamara fma. pygmaea (Tipo)[bc]	578	273, 305	449, 129	n/s	n/s

FN550940 Inocybe fusco-marginata[c]	601	431, 170	281, 320	n/s	n/s
GU980626 Inocybe leucoloma[c]	645	440, 205	289, 356	n/s	n/s
GU980641 Inocybe dulca-mara[bc]	676	n/s	317, 359	n/s	n/s
GU980602 Inocybe fulvipes (grupo A)[c]	689	484, 205	321, 368	n/s	n/s
GU980636 Inocybe dulca-mara[bc]	679	n/s	320, 359	n/s	n/s
FN550936 Inocybe leuco-blema[c]	696	147, 549	325, 371	n/s	n/s
GU980619 Inocybe leuco-loma[c]	680	473, 207	323, 357	n/s	n/s
GU980653 Inocybe arthro-cystis[c]	662	479, 183	307, 355	n/s	n/s
GU980605 Inocybe fulvipes (grupo A)[c]	679	484, 195	321, 358	n/s	n/s
GU980612 Inocybe fulvipes (grupo B)[c]	676	471, 205	321, 355	n/s	n/s
GU980607 Inocybe fulvipes (grupo B)[c]	680	483, 197	321, 359	n/s	n/s
GU980632 Inocybe leucoblema[c]	697	147, 550	325, 372	n/s	n/s
GU980614 Inocybe leucoloma (tipo)[c]	680	474, 206	323, 357	n/s	n/s
HM209795 Inocybe squarrosoannulata (tipo)[c]	695	482, 213	324, 371	n/s	n/s
GU980648 Inocybe cf. terri-gena[c]	664	n/s	314, 350	n/s	n/s
JF908091 Inocybe terrigena[d]	638	n/s	316, 322	n/s	n/s
HQ604783 Inocybe terrigena[d]	694	489, 205	322, 372	n/s	n/s
GU980656 Inocybe fusco-marginata[c]	601	431, 170	281, 320	n/s	n/s
GU980601 Inocybe fulvipes (grupo A)[c]	497	n/s	201, 296	n/s	n/s
GU980654 Inocybe arthro-cystis[c]	625	432, 193	270, 355	n/s	n/s
GU980645 Inocybe dulca-mara[bc]	684	n/s	325, 359	n/s	n/s
FJ904122 Inocybe dulca-mara[bc]	643	n/s	303, 340	n/s	n/s
GU980609 Inocybe fulvipes (grupo B)[c]	646	441, 205	279, 367	n/s	n/s
GU980651 Inocybe arthro-cystis[c]	665	472, 193	311, 354	n/s	n/s
GU980643 Inocybe dulca-mara[bc]	679	n/s	320, 359	n/s	n/s

[a] Kind of study. [b] *Inocybe dulcamara* of GenBank. [c] Sequences of species with names based in the study of Cripps et al. 2010. [d] Sequences obtained of GenBank. n/s = no recognition sites for the enzyme.

al. 2013). The use of molecular techniques could be useful for obtaining specific and precise information for the taxonomic identification of these fungi. Moreover, molecular techniques have been directed at studying changes in the structure and composition of fungal communities, a task difficult to do when only morphological features are used (Gardes & Bruns 1996, Klamer et al. 2002, Osmundson et al. 2013).

In this work, the sequence of ITS achieved for the sample previously identified based on its morphological features as *Inocybe dulcamara* TLXM (despite of showing variations respect to the description by Cripps et al. 2010), had an 84% similarity to the sequence from *I. dulcamara* f. *pygmaea* recorded in the GenBank (Accession number: GU980628) and UNITE. According to the phylogenetic analysis, the Mexican species is related to *Inocybe terrigena*. Nevertheless, the support for this association is relatively low, with only 63.8%. *Inocybe terrigena* can be distinguished from de Mexican species because of the presence of a true persistent ring and necropigmented basidia (Kuyper 1986), and also by its paler color, squarrose pileus and amygadaliform spores (Esteve-Raventós 1998). This suggests it could be an undescribed species of *Inocybe* subgenus *Mallocybe*. Further systematic review of the Mexican species of *Inocybe* subgenus *Mallocybe*, together with geographical and ecological information, is needed for defining and describing their taxonomic affinities with other similar species worldwide, as suggested by Ryberg et al. (2010).

There is no information on the utilization of RFLP patterns for identifying species of *Inocybe* subgenus *Mallocybe*, nor there any data to which we can compare our results. However, the RFLP band pattern obtained after the *in vitro* digestion of the DNA from the voucher collection was confirmed through bioinformatics simulation. This result allows us to do the same simulation for all the other sequences obtained from the GenBank database, with the purpose of comparing their theoretical RFLP pattern with that obtained in the present study. The number and sizes of fragments obtained from the simulated digestion with the four restriction enzymes were different among species. I.e., *Hae*III produced three fragments and *Pst*I two in the Mexican species, but no cleavage sites were found in any other species for these enzymes. Nevertheless, as we noted before when *Hae*III was used, only two bands could be distinguished in the gel, because two of the fragments were almost identical in size. In the case of *Alu*1, the number of cleavage sites varied from zero to two, depending on the species, with the Mexican specimen having two sites. The only other sequence with two cleavage sites belongs to *Inocybe dulcamara* f. *pygmea* (Accession number GU980628), but as it was mentioned before, this sequence can be distinguish from that of the Mexican specimen because of the lack of cleavage sites with *Hae*III and *Pst*I. In the case of *Eco*RI, one cleavage site was found in all the analyzed sequences, which may be in a conserved region of ITS for all the species in the subgenus *Mallocybe*. So, the RFLP band pattern of the Mexican species is different from all the analyzed taxa, taking into consideration these four restriction enzymes. As a consequence, the target sample may represent an undescribed species of *Inocybe* subgenus *Mallocybe*, but it is still necessary to do more taxonomic studies on this fungal genus in Mexico.

Gomes et al. 1999 suggested that the PCR-RFLP of rDNA analysis represents an efficient and powerful method for identifying fungal isolates or for ecological studies.

However, its use can be limited due to polymorphisms that depend on the fragment's size resulting from the action of the restriction enzymes. Therefore, further analysis of the sequence using bioinformatics software may facilitate the solution of the conflicting data resulting from the analysis of gel images, which may achieve variations in the number of bands that do not correspond with the total size of the original amplicons.

In summary, we observed that a polymorphism obtained with the selected restriction enzymes could be a practical tool for studying one species in field conditions when it is introduced as an inoculant in a revegetation programs.

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