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Genotoxic Profile and Morphological Variation of the *Amanita rubescens* Complex: Traditional Knowledge for Safe Consumption in Mexico

Griselda Nallely Hernández-Rico[1], Pablo Octavio-Aguilar[1*], Roberto Garibay-Orijel[2], and Leticia Romero-Bautista[1]

[1]Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo, Mineral de la Reforma, Hidalgo, México. [2]Laboratorio de sistemática y ecología de micorrizas, Instituto de Biología, Universidad Nacional Autónoma de México, Coyoacán, México.
[*]pablo_aguilar9900@uaeh.edu.mx

Abstract Wild mushrooms are important to the nutritional health and economic subsistence of rural populations in Mexico, but inaccurate identification of mushrooms has led to reported cases of poisoning. The aim of this study is to establish genotoxic profiles of mushrooms of the putative *Amanita rubescens* complex and to link those profiles with morphological attributes that suggest a correct identification of mushrooms, in order to prevent poisoning. Several combinations of amplification products (AMA, PHA, POP1, and POP2 genes) were identified in *A. rubescens* fungi sold in traditional markets; these genes are related to the presence of toxic polypeptides and its enzymatic regulators. The sequences correspond to a previously reported toxic gene family (MSDIM). All samples with the complete toxic gene profile presented reddish to dark-brown sporomes; this is the only attribute that visually distinguishes samples with toxic potential. Our results suggest that the mushrooms sold in traditional Mexican markets do not correspond to the *A. rubescens* complex. We conclude that morphological variability allows for identification of edible and inedible mushrooms.

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Introduction

Mushrooms are outstanding for their diversity and traditional uses that provide economic, cultural, and nutritional benefits. As a result, there is abundant traditional knowledge concerning mushrooms' nomenclature, ecology, management, use, conservation, and identification (Bandala et al. 2014; Gry and Anderson 2014; Mariaca-Méndez et al. 2001). Mushroom consumption in Mexico is regionally variable with preferences for some local species that are found in traditional markets: *Amanita* aff. *caesarea*, *A. rubescens*, *Boletus edulis*, *Cantharellus cibarius*, *Lactarius indigo*, *Morchella esculenta*, *Ramaria* sp., *Russula brevipes*, and *Ustilago maydis*, among others (Bandala et al. 1997; Estrada-Martínez et al. 2009; Herrera and Guzmán 1961; Mariaca-Méndez et al. 2001; Montoya et al. 2003, 2014).

Traditional collectors' criteria for identification of edible mushrooms are based on form, color, consistency, habitat, developmental stage, and season (Guzmán 1999; Hernández-Rico 2011; Hung et al. 2015; Jiménez-González et al. 2013; Montoya et al. 2003; Romero-Bautista 2007). However, there have been reported cases of poisoning associated with misidentification by inexpert consumers, who possess inaccurate or insufficient knowledge (Hernández-Rico 2011). In general, identification criteria to distinguish edible from poisonous species seem to rest not on detailed recognition of the second set but precise knowledge of the first (Ruan-Soto 2018).

Some authors suggest that edible mushrooms in the *Amanita rubescens* complex in North America

constitute different taxa than the European species or cryptic (morphologically similar but genetically distinct) species (Tulloss and Lindgren 1994), which probably have different toxic profiles. Most of the toxins in *Amanita* have been studied and described, with α-amanitin (blocks protein synthesis) and phallacidin (hepato- and nephrotoxic), which are both in the MSDIM toxic gene family (Anderl et al. 2012), as identified by their effects and general location at the genus level. However, it is unknown whether the edible amanitas, such as those belonging to the *A. rubescens* complex, have genes associated with the expression of α-amanitin and phallacidin but less toxic allelic variants that make them safe to consume after cooking. In addition, the activation of toxic genes requires the presence of enzymatic regulators (POP1 and POP2) that transform the protoxin into its active form (Luo et al. 2010).

Genetic variation, including toxic genes, usually causes phenotypic changes that can be appreciated at a glance; identifying morphological features that allow the discrimination of toxic and non-toxic genotypes should help to prevent poisoning (Anderl et al. 2012; Cai et al. 2014; Feregrino et al. 2013; Hallen et al. 2007; Kendrick 2000; Lima et al. 2012; Luo et al. 2010). The aim of this research was to identify the presence of genes associated with toxicity in mushrooms of the *A. rubescens* complex sold for human consumption and to associate genotoxic profiles with morphological variation to establish a putative diagnostic attribute that can be used for safe consumption.

Materials and Methods

Sampling

Thirty sporocarps (i.e., the fruiting body of the fungi) of *A. rubescens* specimens were collected from five regions of Hidalgo, Mexico (Acaxochitlan, Huasca, Mineral del Chico, Pachuca, and Omitlan), which include places with and without traditional consumption of these mushrooms (Table 1; Figure 1). The samples were described, and locality, associated vegetation type, and other data were recorded. The biological material was kept in the mushroom collection of the Autonomous National University of Mexico (UNAM).

DNA Extraction

The standardized system of phenol-chloroform extraction, based on a modification of Gardes and Bruns' (1993) method, was used. Dry samples were processed in a laminar flow hood. Amm² tissue fragment was put in a 1.5 µl microcentrifuge tube, submerged in liquid nitrogen, and macerated with a microbiological handgrip inside the tube. The macerate was suspended in 800 ml of CTAB 2x and incubated in a water bath at 65°C for one hour, moving the tubes every 20 minutes. Six hundred ml of chloroform-isoamyl alcohol (24:1) was added, mixed, and centrifuged at 13,000 rpm for ten minutes. Cold isopropanol (0.6 x) was added to the supernatant, mixed gently for a minute, and centrifuged as above. The pellet was cleaned with 500 ml 70% ethanol, dried, resuspended in 60 ml of dH_2O, and stored at -20°C. The quality and concentration of DNA was assayed by spectrophometry (Bio Spectrometer Basic®).

Amplification

Markers specific to α-amanitin (Walton et al. 2004), phallacidin (Hallen et al. 2007), and two regulatory genes named POP (Luo et al. 2010) were used. As positive controls of the amplification, ITS1F, ITS4, and β-tubulin markers were used (White et al. 1990). Total reaction volume was 15 ml, consisting of 8.84 ml of dH_2O, 2.5 ml of 10 x buffer (200 mm Tris–HCl, pH 8.4, KCl 500 mM), 0.16 ml of dNTPs (2 mM), 0.3 ml of $MgCl_2$ (50 mM), 1 ml of each primer (50 mM), 0.75 U of Taq polymerase (Promega®), and 1 ml of DNA sample. The amplification conditions were 94°C for 8 minutes of initial denaturation, 35 cycles at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and 72°C for final amplification. The product evaluation was made by electrophoresis on acrylamide gels at 15%, over 50 minutes at 90 V. PCR products

Table 1 Sample sites description and its traditional use of the *A. rubescens* complex.

Municipality	Locality	Vegetation	Edible	Common name
Acaxochitlan	La Montaña Viviente and Las Terrazas	Pine-oak	No	Crazy fungus
Huasca	Cerro del Zembo	Oak	Sometimes	Unnamed
Mineral del Chico	Mineral del Chico	Cedar	No	Unnamed
Pachuca	San Miguel Cerezo market	Pine-oak	Yes	Chiquita brisket or small cake
Omitlan	Omitlan	Oak	Yes	Chiquita brisket

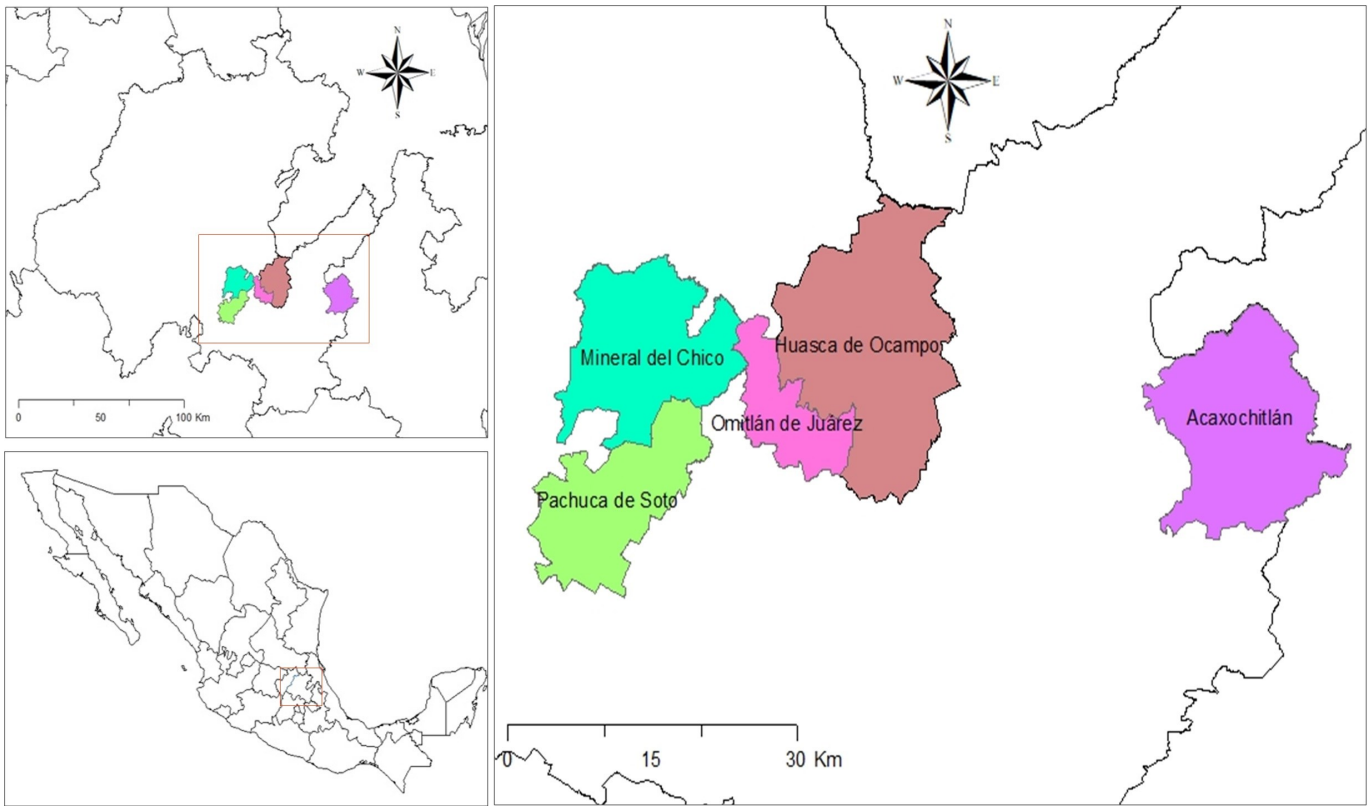

Figure 1 Geographic locations of the sampling sites of *A. rubescens* complex mushrooms.

were used for two subsequent rounds of nested PCR with the primers proposed by Hallen et al. (2007; Supplementary Table 1). Final PCR products of the six samples with a complete genotoxic profile and two probed toxic fungi (*Amanita* aff. *verna* and *A.* aff. *virosa*) were sequenced (Macrogen®), aligned (MEGA 7.0.26; Kumar et al. 2015), and compared with the GenBank database (BLAST-NCBI).

Morphological Analysis

Macro- and micromorphological attributes were described, with continuous (data that can take any value) and discontinuous (variation that can fall into a number of categories or classes) morphological data separated (Table 2). The macromorphological data were 1) pileus (cap): form, color, ornamentation, color of ornamentation, texture (discontinuous data), thickness of the context, diameter, and number of grooves (continuous data); 2) stipe (stem): form, color, ornamentation, color of ornamentation, texture (discontinuous data), thickness of the context, and length and width of the stipe (continuous data); 3) lamella (gills): color, frequency, and edge type (discontinuous data); and 4) ring: form, color, and position (discontinuous data). The micromorphological data were: 1) Melzer´s reaction (amyloid or inamyloid); 2) spore size (length and width); and 3) basidia size (length and width), which are attributes for taxonomic description of mushrooms according to Largent and Baroni (1988).

Analysis

Continuous morphological attributes were used to group the samples based on Ward's method of amalgamation with Euclidean distances. Discontinuous morphological attributes were grouped by simple linkage with Gower's distances, specific to the diverse nature of attributes. The genetic matrix with a specific amplification of genes related to toxicological profile was grouped by UPGMA (unweighted pair group method with arithmetic mean) clustering based on Jaccard's distances (amplified presence-absence data set; Lloyd 2016). For all generated trees, the standard number of groups was obtained by the bootstrap method at 10,000 steps. Paired distance matrix (genetic with Jaccard's distances, morphological by Euclidean distance for continuous data and Gower's distances for discontinuous data) were correlated with a Mantel test to establish a correspondence between the genetic profile and morphological traits, following

Table 2 Categorical traits of the groups formed by the simple linkage agglomerative method of Mexican *A. rubescens* complex. All genotoxic profiles are included in the group 2.

	Group 1	Group 2	Group 3	Group 4
Pileus color	Brown	Reddish-brown	Brown	Brown
Pileus form	Convex	Convex	Convex	Depressed
Pileus ornamentation	Fibrillas	Verruca	Verruca	Without ornamentation
Color of the pileus ornamentation	Reddish-brown	Reddish-brown	Darkbrown	Without ornamentation
Color of the pileus context	Pink	Beige	Beige	Pink
Texture of the pileus context	Spongy	Fleshy	Fleshy	Spongy
Stipe color	Pink	Cream	Cream	Pink
Stipeform	Claviform	Cylindrical	Claviform	Cylindrical
Stipe ornamentation	Fibrillas	Fibrillas	Fibrillas	Fibrillas
Color of the stipe ornamentation	Pink	Reddish*	Pink	Pink
Color of the stipe context	Without color	Pink	Pink	Pink
Texture of the stipe context	Fibrous	Spongy	Spongy	Fibrous
Lamella color	Beige	Cream	Cream	Cream
Frequency of the sheets	Close together	Close together	Close together	Close together
Edge of thelamella	Sawing	Sawing	Fimbriated	Fimbriated
Ring form	Fragile	Membranous	Membranous	Membranous
Ring color	Beige	Cream	Cream	Beige
Ring position	Subapical	Apical	Subapical	Apical
Spores form	Ellipsoids	Ellipsoids	Ellipsoids	Ellipsoids
Spores staining (Melzer's reaction)	Amyloid	Amyloid	Inamyloid	Amyloid

*Exclusive characteristic of Group 2.

transformation of the data with the formula $z = (x - \mu)/\sigma$, where x is the original distance between individual, μ is the average distance, and σ is the deviation of the distance. This transformation was used to orthogonalize the magnitude of the distances.

Results

Molecular analysis grouped the mushrooms in two clades and one isolated individual from Pachuca de Soto with ITS1F as the unique amplified gene (Figure 2a). Individuals in the first group mostly amplified only to the control genes (seven individuals in black ¥), while some individuals presented one or two toxic profile genes (three individuals in orange †). The second group consisted of six individuals from the Pachuca market that amplified to all toxic profile genes and controls (AMA, PHA, POP1, POP2, ITS1F, and β-tubulin) (red ‡), as well as some individuals with black (¥ four individuals) and orange († nine individuals) profiles as described above.

A partial fragment of DNA flanked by the nested primers was obtained for α-amanitin (268.9 ± 17.25, rank 236–287 bp) and phallacidin (96.75 ± 2.94, rank 96–98 bp, Supplementary Table 2) in the six samples with the complete genotoxic profile from Pachuca de Soto (red group) and positive controls (*A.* aff. *verna* and *A.* aff. *virosa*). The first amplified sequence in the red group of *A. rubescens* corresponds to *A. pallidorosea* α-amanitin gene cds (84%, KC778580.1), *A. exitialis* α-amanitin gene cds (71%, KF813063.1), and *A. fulginea* α-amanitin gene cds (71%, KC778575.1). Positive controls (*A.* aff. *verna* and *A.* aff. *virosa* respectively) amplified correspond to the α-amanitin gene from *A. pallidorosea* (84% and 82% identity, KC778580.1), *A. phaloides* (both 79%, KC778577.1), *A. fulginea* (78% and 79%, KF552088.1), and *A. exitabilis* (68% and 79%, KF813063.1).

The second amplified sequence of the red group corresponds to phallacidin (PHA1) partial genes from *A. biosporigera* (identity 86%, EU196141.1), *A. virosa* (84% FN555144.1), and *A. exitalis* (84% KC778564.1). Controls (*A.* aff. *verna* and *A.* aff. *virosa* respectively) correspond to partial phallacidin genes from *A. biosporigera* (82% and 81%, EU196143.1), *A. virosa* (80% and 79%, FN555144.1), and *A. exitalis* (78% and 76%, KF813064.1), all of them poisonous.

The grouping by simple linkage with Gower's distances showed four groups (Figure 2b). All individuals with the complete genotoxic profile are included in the second group. The distinctive feature of the group was the reddish color of the stipe

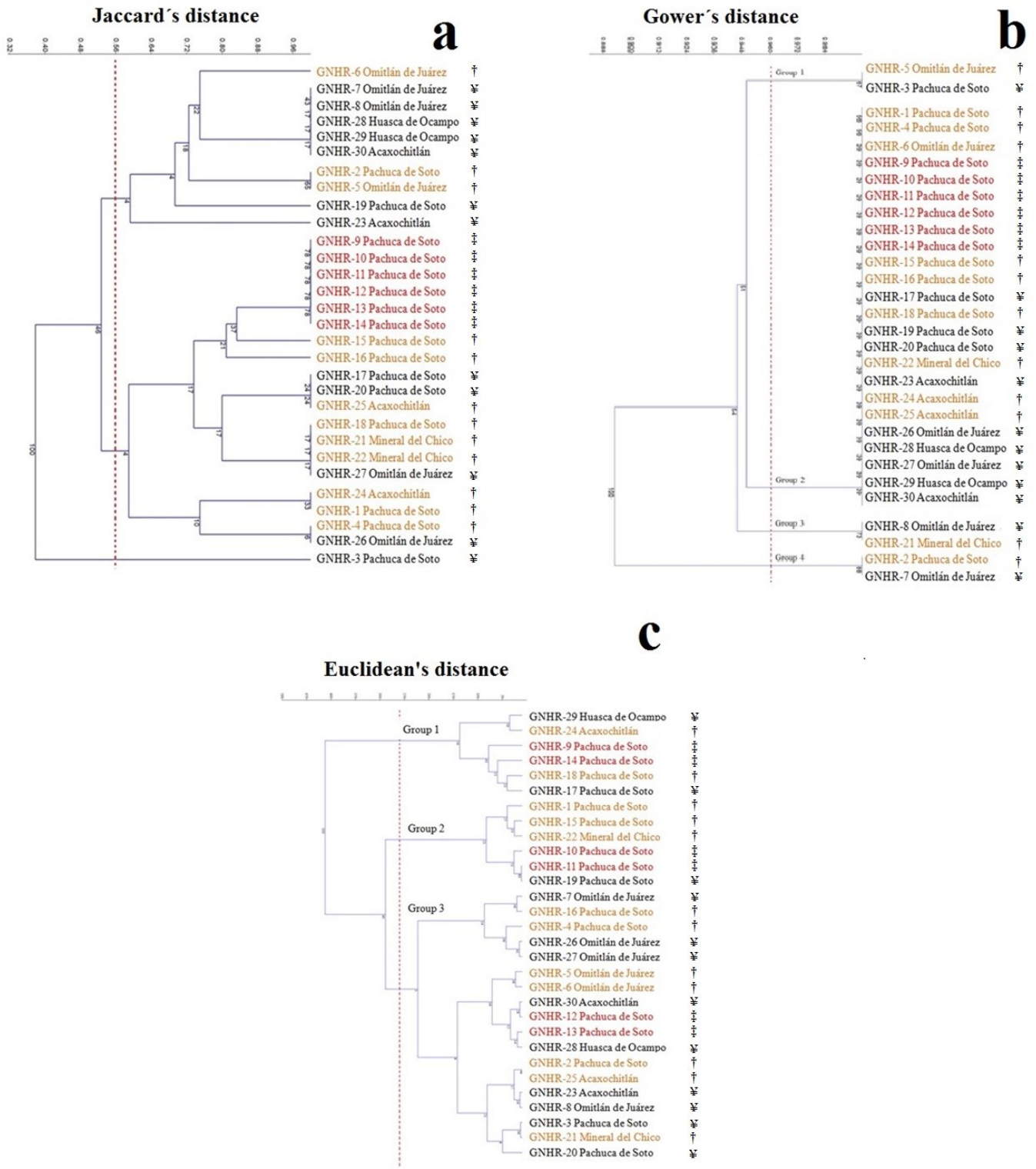

Figure 2 Geographic **A** Molecular grouping using the UPGMA method, **B** simple linkage grouping with Gower's distance of morphological discontinuous data, and **C** ward grouping with Euclidian distances of continuous morphological variables. Red samples (‡) showed all molecular amplifies including genotoxic and positive controls of *A. rubescens* complex in Hidalgo, orange samples (†) showed one to three genotoxic markers, black samples (¥) only showed positive control amplifies.

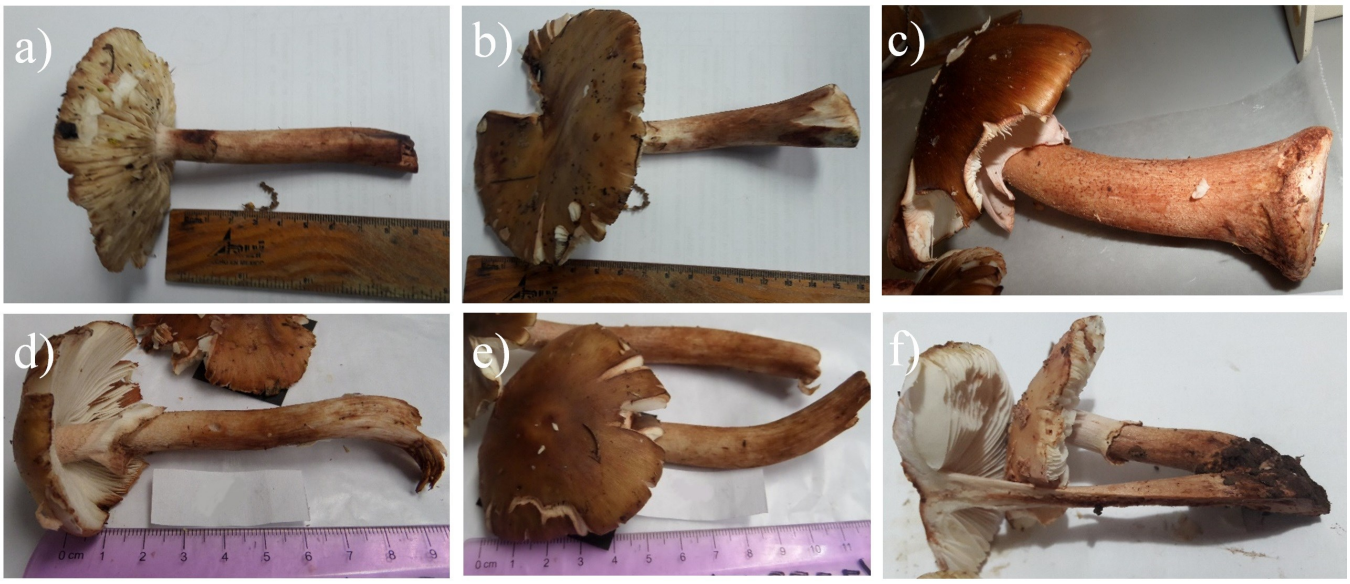

Figure 3 Samples of *A. rubescens* complex with reddish color detail. **A** GNHR-1 (AMA, PHA positive), **B** GNHR-4 (AMA, PHA positive), **C** GNHR-9 (complete genotoxic profile), **D** GNHR-16 (only POP1 negative), **E** GNHR-18 (only PHA negative), and **F** GNHR-29 (no toxic profile). The reddish color of the sample with the complete profile is highlighted (c), the brown color of the samples with a partial genotoxic profile (a, b, d, e) and the light color of the negative sample (f).

ornamentation. In general, all individuals with complete or partial genotoxic profile had a darker reddish coloration (Table 2; Figure 3).

The Ward's grouping showed three morphological groups associated with sporome size (Figure 2c). The smaller mushrooms are grouped in the third group, although they have wide stretch marks on the pileus; the largest mushrooms are grouped in the second group, and do not have stretch marks on the pileus. The first group is made up by medium size mushrooms (Table 3). However, none of these morphological groups are related to the genotoxic

Table 3 Description of the groups formed with Euclidean distances obtained from continuous traits of the *A. rubescens* complex.

		Group 1	Group 2	Group 3
Macromorphology	Minimum diameter of the pileus (mm)	48.2 ± 16.22	78 ± 24.97	6.94 ± 20.2
	Maximum diameter of the pileus (mm)	81.8 ± 27.2	102.5 ± 19.89	80.67 ± 23.1
	Stretch length (mm)	0.8 ± 2.04	0	1.06 ± 1.9
	Context of the pileus (mm)	6 ± 1.79	5.67 ± 0.52	6.11 ± 3.4
	Minimum longitude of the stipe (mm)	91.5 ± 39.24	0	0
	Maximum longitude of the stipe (mm)	114.2 ± 32.31	92.5 ± 18.06	70.5 ± 42.1
	Minimum width of the stipe (mm)	13.2 ± 3.71	0	0.72 ± 3.1
	Maximum width of the stipe (mm)	13.8 ± 10.09	14 ± 2.1	13.22 ± 5
	Context of the stipe (mm)	16 ± 4.69	14 ± 2.1	13.22 ± 5
Micromorphology	Minimum longitude of the spore (µ)	7.4 ± 0.92	7.33 ± 0.5	7.4 ± 0.5
	Maximum longitude of the spore (µ)	10.4 ± 1.07	9.73 ± 0.25	10.43 ± 1
	Minimum width of the spore (µ)	5.9 ± 0.46	5.72 ±0.44	5.64 ± 0.5
	Maximum width of the spore (µ)	8 ± 0.84	7.92 ± 0.48	7.81 ± 0.8
	Minimum longitude of the basidia (µ)	25.7 ±2.31	22.57 ±1.76	21.22 ± 6
	Maximum longitude of the basidia (µ)	35.3 ± 2.55	32.87 ± 2.37	31.16 ± 8.7
	Minimum width of the basidia (µ)	9 ±0.49	7.58 ± 1.45	7.27 ± 2
	Maximum width of the basidia (µ)	11.7 ± 0.84	10.28 ± 1.32	10.07 ± 2.7

profile. Finally, the Mantel test showed no correlation between genetic and morphological distances.

Discussion

Our results show that edible fungi from Hidalgo markets are different from the European *A. rubescens* complex because they show high variability in the presence or absence of genes associated with toxicity, which are totally absent in *A. rubescens* sensu stricto as defined based on the European taxa. One group from Pachuca was positive for all analyzed genes (AMA, PHA, POP1, POP2, and the positive controls ITS1F and β-tubulin) that correspond to the sequenced toxic genes in other *Amanita* species of the section Phalloidae. Several samples have different combinations of analyzed genes, but these genotypes showed no clear relationship with morphological variation. However, qualitative morphological analysis showed that the reddish to dark-brown color of sporomes is a possible attribute associated with a partial or complete genotoxic profile.

The presence of genes responsible for the expression of toxins in the *A. rubescens* complex suggests that they are potentially dangerous; the complex is accordingly assigned to section Phalloidae, where most species are toxic (Cai et al. 2014). The variability of combinations in genotoxic profiles could relate to deletions and/or duplications in the copy number of the sequences AMA and PHA, which give rise to hypervariable regions that code for different peptides of between seven to ten amino acids (Hallen et al. 2007). However, we cannot assume that the fungi really are toxic, as it is unknown which genes are expressed and to what extent. To elucidate toxicity, assays by liquid chromatography mass-spectrometry (Parnmen et al. 2016) for the detection of expression products are necessary.

The fact that the AMA and PHA genes are absent in non-toxic fungi in section Phalloidae (Hallen et al. 2007) suggests that the mushrooms consumed in Hidalgo are mistakenly identified as species within the *A. rubescens* complex, or that some edible non-toxic mushrooms in section Phalloideae conserve the potential to express toxicity. In addition, fungi can have other compounds that result in poisoning in combination with alcoholic beverages or some foods. For example, *Coprinus atramentarius* has coprine, which in combination with alcohol causes pain and sickness (Graeme 2014; Gry and Andersoon 2014; Jo et al. 2014). In particular, *A. rubescens* presents a thermolabile toxin called rubescenslysin, which interacts with phospholipids in cell membranes, generating intracellular hemolysis, cardiotoxicity, and adverse effects in the central nervous system. The sequence of genes associated with this toxin is not yet known, so no appropriate molecular markers are available (Odenthal et al. 1982; Seeger and Wachter 1980).

With respect to the categorical traits associated with the presence of AMA, PHA, POP1, and POP2 genes; the distinctive attribute for recognizing a potentially toxic mushroom is the dark reddish stipe ornamentation (Table 2; Figure 2b). The other five recorded traits are shared between groups that present partial genotoxic profiles. However, in general, it could be said that more intense reddish-brown-colored mushrooms tend to have more genes associated with toxicity.

On the other hand, morphological variation of the continuous characteristics did not show any structure that would allow for the identification of toxicity. Traditional sellers group mushroom according to size for pricing; attributes such as size, width, and weight that are traditional criteria of the vendors do not appear to be related to toxicity (Burrola-Aguilar et al. 2012; Hernández-Rico 2011; Rodríguez-Muñoz et al. 2012).

Conclusions

Women are the main fungal collectors in the central mountainous region of Hidalgo. In general, the criteria for choosing edible fungi vary across regions, so some cases of mycetism in Acaxochitlan and Real del Monte have removed *A. rubescens* from local diet. Our results suggest modifying the traditional criteria for the selection of edible fungi, those similar to *A. rubescens*, by considering the color intensity. In addition, the possibility of horizontal transfer of MSDIM genes and/or the preservation of toxic potential in the genome of *A. rubescens* (from Hidalgo, at least), brings into question the viability of continuing consume this fungus.

In conclusion, these results show that a macromorphological characteristic, color, is associated with a complete or partial genotoxic profile among edible fungi identified as *A. rubescens*. This is not yet a solid guideline for the collection of guaranteed edible mushrooms. In order to develop more conclusive recommendations for traditional mushroom collectors and vendors, these results must be replicated using a larger sample size, and the evaluation of toxicity must

considerer traditional collectors' criteria used to form morphological groups. However, these results are a significant contribution to developing restrictive criteria (intense reddish-brown-colored mushrooms) for the avoidance of potentially toxic fungi.

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Declarations

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