Published in final edited form as:

J Ethnopharmacol. 2012 November 21; 144(2): 371–378. doi:10.1016/j.jep.2012.09.022.

DNA Barcoding of the Mexican Sedative and Anxiolytic Plant *Galphimia glauca*

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Abstract

Ethnopharmacology relevance—*Galphimiaglauca* (Malpighiaceae) is a Mexican plant popularly used as a tranquilizer in the treatment of nervous system disorders, although it is also used to treat other common illnesses.

Aim of the study—The aim of this investigation is to find out if populations of *Galphimiaglauca* collected in different regions and ecosystems in Mexico actually belong to the same species by using the contemporary technique of DNA barcodes. Our previous metabolic profiling study demonstrates that different collections of this plant obtained from various geographical areas exhibited diverse chemical profiles in terms of the active compounds named Galphimines. We expected the DNA barcodes apart from indicating the different species of *Galphimia* would indicate the active populations.

Materials and methods—We employed *matK*, *rpoC1* and *rbcL* DNA barcodes to indicate the different species. Furthermore to investigate the possible impact of the several different ecosystems where the seven populations were collected, thin layer chromatography was employed to create a partial chemical profile, which was then compared with the metabolic profiles obtained by ¹H-NMR and multivariate data analysis.

Results and conclusions—This study showed that the seven populations here analyzed contain at least three different species of the genus *Galphimia*, although each individual population is homogeneous. Interestingly our TLC analysis clearly showed that the active populations displayed a distinctively unique chemical profile. This work also showed that the use of DNA barcodes combined with chemical profile analysis is an excellent approach to solve the problems of quality control in the development of *Galphimia*-based medicines, as well as for any breeding programs for this species.

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Keywords

DNA barcodes; *Galphimiaglauca*; sedative; anxiolytic; Galphimines

1. Introduction

Galphimiaglauca (Cav.) Kuntze (Malpighiaceae) is a tropical and sub-tropical plant that grows in Mexico and is commonly known as “calderonaamarilla”. This plant is a popular herbal medicine which has been used as a tranquilizer in the treatment of nervous system disorders since prehispanic times (Estrada, 1985). A family of nine bioactive *nor*-secofriedelanes with sedative and anxiolytic properties, named galphimines, were identified in specimens of *Galphimiaglauca* growing in the locality of Doctor Mora, Guanajuato (Cardoso-Taketa et al., 2004). The sedative activity and the anxiolytic action of galphimines have been documented using *in vitro* models (Prieto-Gómez et al., 2003; Herrera-Ruiz et al., 2006). In a clinical study performed with subjects exhibiting symptoms of general anxiety, standardized preparations of *Galphimiaglauca*, administered to the patients produced anxiolytic effects (Herrera-Arellano et al., 2007). This plant has also been found to produce other pharmacological effects, including anti-inflammatory, anti-malaric, anti-allergic and anti-asthmatic activities (Diaz, 1976; Dorsch et al., 1992; Neszmelyi et al., 1993; Camacho, 1997; Sharma et al., 2012). *Galphimiaglauca* has been commercialized internationally and made available in several online herbal drug stores.

Our two previous metabolic profiling studies of *Galphimiaglauca* using ¹H-NMR spectroscopy and multivariate data analysis, clearly showed differences in metabolic profiles of *Galphimiaglauca* populations in levels of sedative, anxiolytic and anti-inflammatory activities that were clearly related to the region of collection (Cardoso-Taketa et al., 2008; Sharma et al., 2012). Two of the populations (GM and QJ) were particularly high in galphimines (active populations), whereas all of the other populations (MM, MT, MC, JG and CT) did not seem to have detectable levels of these secondary metabolites. Interestingly, the anti-inflammatory activity was found to be high in all populations (Sharma et al., 2012). The presence of galphimines in the GM and QJ populations and the absence in the MM, MT, MC, JG and CT samples, has also been confirmed via high-performance liquid chromatography (HPLC) analysis. Partial least square regression discriminant analysis (PLS-DA) confirmed a positive correlation between the presence of galphimines and the sedative and anxiolytic responses displayed by those extracts (Cardoso-Taketa et al., 2008; Sharma et al., 2012). The differences seemed to indicate that the chemical profiles exhibited among the *Galphimiaglauca* populations collected in the different regions of Mexico, could be related to geographical, climatic, or soil conditions.

A recent taxonomic study reported that the genus *Galphimia* Cav. comprises 26 species, most of which are found in Mexico (Anderson, 2007). Due to the similar phenotype of these plants, the identification of each of the species is confused and difficult, resulting in, many collectors labeling all specimens as either “*Galphimiaglauca*” or “*Galphimiagracilis*” (Anderson, 2007). In our recent study (Sharma et al., 2012) all populations collected from seven specific locations over a wide area in Mexico were identified as *Galphimiaglauca* in

the Mexican Herbarium “HUMO Herbarium”, CEAMISH (Centro de Educación Ambiental e Investigación Sierra de Huautla), UAEM, Cuernavaca.

With the use of standardized DNA sequencing it was assumed that universally amplified, short and highly variable regions of DNA could be employed as DNA barcodes to identify species with a high degree of exactness (Herbert et al., 2003). An ideal DNA barcode should contain enough variations to detect the differences in closely related species and should have highly conserved regions for easy sequencing and editing by a single pair of primers. In some groups of animals this has been achieved to some extent with the use of *cytochrome c oxidase I* (CO1) (Floyd et al., 2002; Herbert et al., 2003). However, in plants, amplifying a single barcode is not enough to differentiate between closely related species. In recent years several regions of the plastid genome have been proposed and validated as leading candidates for viable DNA barcodes in plants. Four of them are coding genes (e.g. *rbcL*, *rpoC1*, *matK*, *rpoB*) and four others are non-coding spacers (*psbA-trnH*, *trnL*, *atpF-atpH*, *psbK-psbI*) (Kress et al., 2005; Pennisi, 2007; Kress and Erickson, 2007; Chase et al., 2007; Taberlet et al., 2007; Lahaye et al., 2008; Ledford, 2008; Ford et al., 2009). Recently, the Consortium for the Barcoding of Life (CBOL) Plant Working Group reached consensus and recommended the use of the *matK/rbcL* combination as a universal DNA barcode for land plants (CBOL Plant Working Group, 2009). The authors indicated that the discrimination success rate of these markers was up to 72% in the cases tested. The few studies that have been focused on the molecular systematics of several genus and species of the Malpighiaceae family, used the molecular markers: *matK*, *rbcL*, *ndhF* and *PHYC*, three plastidic and a nuclear barcode, respectively (Cameron et al., 2001; Tokuka and Tobe, 2006; Davis and Anderson, 2010).

The aim of the present work was to determine through the use of DNA barcoding, employing *matK*, *rbcL* and *rpoC1* genes, if the specimens of “*Galphimiaglauca*” collected in the seven different regions and ecosystem in Mexico (Sharma et al., 2012) belong to the same species, or if they belong to various species according to the taxonomic study of Anderson for plants of the genus *Galphimia*.

2. Materials and methods

2.1 Plant material

Specimens were collected from seven different localities in Mexico: MC (Cuernavaca, Morelos), MT (Tepoztlán, Morelos), GM (Doctor Mora, Guanajuato), JG (Guadalajara, Jalisco), QJ (Jalpan, Querétaro), CT (Tuxtla Gutiérrez, Chiapas), and MM (Miacatlán, Morelos). Six individuals were collected from each population in the summer of 2009 (August and September). Specimens were deposited at the HUMO Herbarium, CIByC (Centro de Investigación en Biodiversidad y Conservación), UAEM, after authentication by herbarium personnel. Details for plant specimen collection are presented in Table 1. These same samples were used to perform a metabolic profiling study recently published (Sharma et al., 2012), and also for the investigation reported herein. The samples were kept at -20°C until used for DNA extraction.

2.2 DNA extraction

Genomic DNA was isolated from frozen young leaf tissue (100–130 mg) using the Plant/Seed DNA Isolation Kit (Zymo Research) according to the manufacturer's protocol. Nucleic acids were quantified using a NanoDrop™ by measuring the absorbance at 260 and 280 nm. DNA quality was also examined by 1.0% agarose gel electrophoresis. DNA samples were diluted with nuclease free water into working stock solutions (20 ng/μL), and stored at –20°C before use.

2.3 Oligonucleotides

The following oligonucleotides, *rbcL* (RF2-GAAGGGTCTGTTACTAACATG,RR3-TCCCCTTCAAGTTTACCTAC)and *rpoC1* (1F-GTGGATACACTTCTTGATAATGG,4R-CCATAAGCATATCTTGAGTTGG) previously reported by Tokuoka and Tobe 2006 as well as by the Royal Botanic Garden, respectively, were used. Four combinations of primers to amplify *matK* available on Royal Botanic Garden's website were initially employed, however, they showed weak or non-specific amplification results in PCR assays. To obtain more specific results, new primers were designed for *matK* (F-GAGGGGTTTGCAGTCATTGT, R-CCAATGACCCAATCAAAGGA) by using highly conserved regions of available sequences in GenBank. These new primers were designed using the Primer 3.0 software.

2.4 PCR amplification, purification and sequencing

For all three markers, Polymerase Chain Reaction was performed in a Robocycler gradient 96 (Stratagene) using the following program: An initial step of 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, followed by a final extension of 10 min at 72°C. PCR was performed in a 25 μL reaction volume using “Ready to go PCR beads” (GE Life Sciences) according to the manufacturer's protocol. To each tube containing PuRe Taq DNA Polymerase, dNTPs 200 mM in 10 mM Tris-HCl pH 9.0, 50 mM KCl, and 1.5 mM Mg Cl₂, water was added to standardize a final volume of 25 μL. About 1 μL of template DNA and 1μL of each primer (10 pmol/μL) were added separately to each reaction. PCR products (1μL each sample) were checked on 2.0 % agarose gel electrophoresis to confirm the amplification of the desired product. The PCR products were next purified using the QIA quick PCR Purification Kit (Qiagen) according to manufacturer's directions. About 1 or 2 μL of the PCR product was then quantified with a NanoDrop™. The concentration of purified PCR product was adjusted to 1kb=100 ng/μL before sequencing.

All markers were sequenced in both directions. For DNA sequencing, 1.4 μL template DNA and 1.0 μL primer (4 μM) were added to 1 μL BigDye 3.1 reaction mixture (Applied Biosystems). To bring the volume to 6.4μL, 1 μL sequencing buffer and water were added. The cycling was at 96°C for 10 sec, 50°C for 5 sec and 60°C for 3 min. After cycling, water was added to each tube to increase the volume to ~25 μL. Then the reactions were purified using Sephadex G50 superfine in 96-well filter plates, and the collected samples were run on an Applied Biosystem 3730XL capillary instrument (DNA Sequencing Facility, University of Chicago Cancer Research Center). All sequences were visually inspected and edited using Sequencher 4.8 (Gene Code Corp.). Based on the 6 forward and 6 reverse sequences, a

consensus sequence was obtained for each population. All consensus sequences were deposited in GenBank (see Table 3 for the accession numbers).

2.5 Phylogenetic analysis

All available sequences for *rbcL* and *matK* deposited in GenBank for the genus *Galphimia* were incorporated in this analysis to investigate their relationship (Table 2). In addition, some sequences from other related genus were also incorporated for phylogenetic out groups (Table 2). We did not find any reported DNA sequence in GenBank for *rpoC1* in the case of *Galphimia*. The sequences were aligned using Clustal W (Higgins et al., 1994) with default parameters, and the phylogenetic analysis was performed in MEGA 5.05 (Tamura et al., 2011). For each marker we generated separate Neighbour-joining (NJ), Maximum likelihood (ML) and Maximum parsimony (MP) phylogenetic trees (results for ML and MP are shown in Supplementary material) based on Kimura's 2-parameter (K2P) nucleotide evolution model (Kimura, 1980), data specific model (Nei and Kumar, 2000) and close-neighbor-interchange algorithm (Nei and Kumar, 2000), respectively. The sequences for all three markers were combined with a total length of 2067 bp, and phylogenetic analysis was done using the NJ method. Node support was assessed via 1,000 bootstrap replicates. We employed NJ, ML and MP methods as they have superior computationally intensive analysis and because some studies have shown that the choice of phylogenetic method algorithm did not change DNA barcode results (Elias et al., 2007; Lahaye et al., 2008).

2.6 Thin layer chromatography

Methanolic extracts were prepared for all seven populations (five individual extracts for each population except for MM where four individual extracts were employed). The samples (leaves) were dried for 3–4 days in a cool and dry place without direct sunlight. Samples were then pulverized by mortar and pestle. Pulverized dried material (100 mg) for each sample was mixed with 1 mL MeOH. Samples were vortexed for 2 min, sonicated for 15 min and then centrifuged at 10,000 rpm for 15 min. The supernatant was removed and the material residue containing the pellet was reprocessed five times. To achieve an exhaustive extraction all supernatants were combined, applied directly in thin layer chromatography (TLC) using a mobile phase of CHCl_3 : $\text{CH}_3\text{COOCH}_2\text{CH}_3$ (2:1 v/v). TLC sheets were stained with a spray solution containing vanillin (1 g of vanillin in 100 mL of H_2SO_4), and slightly heated until the colors appeared.

2.7 Partial least square regression modeling-discriminant analysis (PLS-DA)

The SIMCA-P 11.0 software (Umetrics) was used to perform partial least square regression modeling-discriminant analysis based on ^1H -NMR spectra of all the methanolic crude extracts derived from plant samples, resulting in “active” and “inactive” anxiolytic and sedative populations labeled into two classes. Such spectra were also used in our previous work (Sharma et al. 2012) to perform a metabolic profiling study.

3. Results and discussion

The primary objective of this study was to investigate the genetic variability of populations of the medicinal plant *Galphimiglauca* collected from different geographical locations in

Mexico. In this work, we have employed molecular markers to test the discrimination power of each of these three DNA barcode regions. Genomic DNA extracted from CT samples and typical PCR amplicons obtained for the three DNA barcodes here used are shown in the supplementary material section. Amplification of *matK*, *rpoC1* and *rbcL* DNA barcodes for all *Galphimia* populations here analyzed yielded alignments of 805bp, 599 bp, and 665bp, respectively. All six individuals of each population were found to be 100% identical at the DNA level. The sequences were highly similar among members of each population but yet sufficient single nucleotide polymorphisms were observed among populations in the case of the *matK* and *rpoC1* genes. Based on these alignments, three bootstrap consensus trees were constructed. All phylogenetic trees had strongly supported clades with high bootstrap values. Three major clades were formed for all three markers, and all seven populations were separated in three well supported sister clades in the case of *matK* and *rpoC1* (Figs.1 and 2). These three sister clades based on the two markers allowed us to distinguish active populations (GM, QJ) from inactive ones (MM, MT, MC, JG, CT). The *rbcL* region did not show sufficient variations to discriminate active populations from inactive ones (Fig. 3). The CT samples presented the highest rate of polymorphism among all, as CT was clearly separated from other populations in all three phylogenetic trees based on *matK*, *rpoC1* and *rbcL*(Figs. 1–3). The added sequences from the database were separated into two major clades including highly similar and dissimilar sequences for all three DNA barcodes. The bootstrap consensus trees resulting from the Maximum Likelihood method of these three DNA barcodes, was highly similar with the trees of the NJ analysis. The bootstrap values for ML and NJ were also highly similar for all the trees. The ML method recovered all three major clades and three sub-clades. Results are shown in Supplementary material. The combined sequences analysis fully supported the results obtained by individual marker analysis (*matK* and *rpoC1*) as shown in Fig. 4. In some cases, when sequences were identical, bootstrap values, were comparatively lower than when sequences were not identical. In this phylogenetic analysis all seven population were clearly separated into three distinct groups; 1) active populations (GM, QJ), 2) inactive populations (MM, MT, MC, JG) and 3)CT, also an inactive population. These results indicate that the populations here analyzed do not belong to one species of *Galphimia* but possibly to at least three different species of the genus *Galphimia*. Additional botanical studies are needed to confirm the results of our molecular analysis, however, these studies are actually outside the boundaries of this investigation. These findings may answer the question of why populations collected from different localities in Mexico exhibit diverse chemical profiles and different biological activities (Taketa et al., 2008; Sharma et al., 2012).

Several DNA sequences of *matK* and *rbcL* deposited in GenBank for the genus *Galphimia* were incorporated in this analysis in order to investigate their relationship with the samples here studied. Additional sequences belonging to other genus related to *Galphimia* were also chosen to construct out groups (accession numbers are given in Table 2). In the case of *rpoC1* we found no other data deposited in the database for the genus *Galphimia*, so only similar and highly dissimilar sequences were chosen for BLAST analysis (accession numbers are given in Table 2).We also employed the Maximum Parsimony (MP) method to analyze the data but this algorithm did not alter the results (see supplemental materials), and clade separation was the same for all tested methods (NJ, ML, and MP). Most of the

sequences of *matK* and *rbcL* for the genus *Galphimia* were deposited by Davis and Anderson in 2010, but accession numbers were not been given to all plants reported in the article, and therefore they are not available in GenBank until now, and could not be used for this phylogenetic analysis. In their study, such sequences were classified according to recent taxonomy data (Anderson, 2007), but two sequences for *Galphimiaglauca* (accession numbers AB233800 for *matK* and AB233904 for *rbcL*) had been deposited before the taxonomy study was done. Although these sequences were labeled as “*Galphimiaglauca*”, it is difficult to say if they were identified according to the current taxonomic data or misinterpreted for other related species of the genus, because according to Anderson, 2007 samples growing in the region of Doctor Mora, Guanajuato (GM samples) were *Galphimiaglauca*. However, our GM samples proved to be genetically quite different from than previously reported as *Galphimiaglauca* when subjected to phylogenetic analysis on the basis of both *rbcL* and *matK* sequences. Our CT samples, on the basis of 100% sequence identity with previously reported sequences of *matK* and *rbcL* indicate that these samples may belong to the species *Galphimiaspeciosa* (Figs. 1 and 3). Davis and Anderson (2010) have given no explanation as to why the sequence of *Galphimiamirandae* was found identical to *Galphimiaspeciosa*

The phylogenetic tree obtained by the NJ method based on combined data analysis shows highly similar results with those obtained by each of the individual markers. The objective behind using separate and combined sequence data was to analyze the discrimination capacity of each marker in this genus and the consistency of the phylogenetic trees. As we have demonstrated in previous studies, metabolic profiling is a powerful approach to study the chemical profile of *Galphimia* populations (Cardoso-Taketa et al., 2008; Sharma et al., 2012). We have shown that galphimines are good markers for central nervous system activity and screening purposes. In our previous work we assumed that environmental factors due to the geographical locations where these plants were grown, could lead to the production and accumulation of different metabolites. The results obtained in the present investigation suggest that genetic factors are playing an important role in determining what compounds would be produced and at what level. The data here presented suggest that these seven populations belong to a minimum of three different species and open the possibility for future botanical studies to confirm or deny by taxonomical data the results obtained through our methods of investigation. In order to compare those results with the ones of the chemical profile, we applied a simple but yet efficient approach using thin layer chromatography (TLC). A partial chemical TLC profile was obtained for the methanolic crude extracts prepared from the individuals of the seven populations. This assay revealed that each profile was unique and differed from all of the others, and that active and inactive populations showed clearly different chemical profiles on the basis of the amount and color of the metabolites detected (Fig. 5). The active populations (Fig. 5 marked as GM and QJ) present a galphimines profile in black rectangle, whereas inactive populations are further divided in two categories, inactive population with blue colored compounds (marked as MM, MT, MC, JG) and other inactive population without blue colored compounds (CT). Interestingly, results show that the phylogenetic tree generated by DNA data is fully supported by the TLC chemical profiles of these plant specimens (Fig. 5). We have not yet identified all compounds detected by TLC, but the consistency observed in the chemical

profiles of each population was uniform and stable. Fig. 5 compares a bootstrap consensus tree based on *matK*, with the TLC chemical profiles of the samples here studied. Thus, TLC profiles were efficient to distinguish active and inactive populations and this method can be used as a quick screening tool to distinguish active from inactive samples.

The TLC profiles here presented were highly reproducible and stable, and as such they are a discriminating factor among populations. A visual inspection of the TLC sheets showed that active populations (QJ, DM) and inactive ones (MM, MT, MC, JG, and CT) contain different types of secondary metabolites. Those blue colored compounds present in the inactive populations share a partially similar profile for MM, MT and JG populations although the amounts of these compounds vary as to higher and lower concentrations, except for MC, where these compounds are hardly present. Each observed metabolite was found in all the individuals of the same population, in higher or lower concentration, presenting a consistent chemical profile per population.

It is known that a single amino acid change in the coding region may exhibit important effects in the metabolism of any living organism. In these plant populations genetic similarity and dissimilarity may have contributed to the production of particular metabolites as observed in their TLC profiles. However, environmental factors cannot be ignored as all populations have been collected from different geographical location, i.e., MC population showed a chemical profile differing from the others of the same group (inactive populations with blue colored compounds). CT, another inactive population, exhibited a distinctly different chemical profile, while the active populations GM and QJ were found to be major producers of galphimines.

The population CT differed genetically from the rest of the populations according to all DNA barcode markers as well as in its TLC chemical profile because of the absence of galphimines and blue colored compounds. Our study indicates that although genetic factors may be primarily responsible for the production of secondary metabolites in these plants, environmental factors (e.g., soil and climatic conditions) may also play an important role as indicated in the case of MC and MM, MT, JG populations. The TLC chemical profiles and phylogenetic analysis clearly demonstrated the genotype and phenotype relationship where the three sister clades of phylogenetic tree represented three different groups of plant populations with distinct chemical profiles. In the group of samples showing blue colored compounds, the MC population stands out for its near lack of any blue colored compound, but did not show any other genetic difference from the other members of this group.

A metabolic profiling using partial least square regression modeling-discriminant analysis based on ¹H-NMR or in TLC profiles PLS-DA clearly separated all studied populations into two groups (Fig. 6). Group 1 contained only active populations (GM and QJ) and group 2 consisted of all inactive populations (MM, MT, MC, JG and CT). In the group of inactive populations, CT and MC further differed within the group by lacking blue colored compounds as suggested in their TLC chemical profiles. It is important to note that a simple approach (TLC) as in this study and a complex approach (NMR) yielded similar results. It should be noted that in the TLC profile, the MC population only exhibited some chemical

compounds which have agreement with the mobile phase system used, and NMR represents a much more complete chemical profile.

We had successfully distinguished plant specimens of the genus *Galphimia* using DNA barcodes as a tool. The phylogeny deduced from the DNA barcode data matched quite closely to the TLC chemical profiles and to ¹H-NMR based PLS-DA profiles. This study suggests that plant DNA barcodes, TLC and metabolic profiling results in a more clearly defined assessment for quality control and plant breeding programs. Although at this point we cannot define the relationship of the different chemical marker colors to the structure of the compounds, chemotaxonomic studies may prove to be useful for the relationships that match those determined by DNA barcodes data for future applications.

Acknowledgements

This work was supported by Consejo Nacional de Ciencia y Tecnología Mexico (Grant 80980). A. Sharma is indebted to Secretaría de Relaciones Exteriores México, for the doctoral fellowship awarded. This study was also supported in AL Laboratory with funds from the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000, and a sub-award from the Arkansas INBRE program [National Center for Research Resources (5P20RR016460-11) and the National Institute of General Medical Sciences (8P20GM103429-11) from NIH].

Abbreviations

MC	Cuernavaca, Morelos
MT	Tepoztlán, Morelos
GM	Doctor Mora, Guanajuato
JG	Guadalajara, Jalisco
QJ	Jalpan, Querétaro
CT	Tuxtla Gutiérrez, Chiapas
MM	Miacatlán, Morelos

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Fig. 1. Bootstrap consensus tree generated by the Neighbor-Joining method for the *matK* sequences obtained for *Galphimia* populations here studied. Evolutionary analyses were conducted in MEGA 5.05. Numbers below the branches are bootstrap values expressed as percentage of 1,000 replicates.

Fig. 2. Bootstrap consensus tree generated by the Neighbor-Joining method for the *rpoC1* sequences obtained for *Galphimia* populations here studied. Evolutionary analyses were conducted in MEGA 5.05. Numbers below the branches are bootstrap values expressed as percentage of 1,000 replicates.

Fig. 3. Bootstrap consensus tree generated by the Neighbor-Joining method for the *rbcL* sequences obtained for *Galphimia* populations here studied. Evolutionary analyses were conducted in MEGA 5.05. Numbers below the branches are bootstrap values expressed as percentage of 1,000 replicates.

Fig. 4.

The bootstrap condensed tree generated by the Neighbor-Joining method for *Galphimia* populations collected in this study in comparison with *Galphimia* species previously reported in GenBank based on *matK*, *rpoCl*, and *rbcL* combined sequences. The number in each branch shows the bootstrap support of the branch using 1,000 replicates.

Fig. 5.

Comparison of TLC profiles of methanolic extracts from *Galphimia* populations here studied with NJ tree of these samples based on *matK* sequences. Active populations with galphimines are marked with black circles (●), whereas inactive populations with blue colored compounds are marked with black squares (■), while an inactive population (CT) is indicated with with a black triangle (▲). Galphimines are highlighted with a black rectangle. The TLC mobile phase was prepared with $\text{CHCl}_3:\text{CH}_3\text{COOCH}_2\text{CH}_3$ (2:1 v/v). The individuals of each population are numbered (1–5), where B is a purified galphimine B (control) and C is a crude methanolic extract from the GM population (control). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article).

Fig. 6.

Results of the Partial Least Square Discriminant Analysis (PLS-DA1 vs PLS-DA2) based on ^1H -NMR spectra of methanolic crude extracts of all plants here studied. Populations were separated into two groups: Active samples (with galphimines) and inactive samples (without galphimines). Moreover, the inactive group can be divided further into three subgroups 1; CT population (no galphimines and no blue compounds), 2; MC population (some blue colored compounds), 3; MM, MT and JG populations (blue colored compounds). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article).

Table 1

General data for the studied *Galphimia* populations collected in September, 2009.

Population	Voucher	Locality	Date and time of collection	Position	Altitude (m)	Ecosystem
GM	15189	Doctor Mora, Guanajuato	September 16 12.00–13.00 h	W 100° 19.22 N 21° 08.74	2,120	Semi-dry secondary vegetation
MT	15485	Tepoztlán, Morelos	September 6 12.00–13.00 h	W 99° 06.97 N 18° 59.35	1,700	Seasonal –dry tropical forest secondary vegetation
MC	15011	Cuernavaca, Morelos	September 10 13.00–14.00 h	W 99° 13.48 N 18° 58.91	2,204	Pine oak forest
MM	15426	Miacatlán, Morelos	September 25 14.00–15.00 h	W 99° 21.75 N 18° 45.57	1,004	Seasonal–dry tropical forest secondary vegetation
JG	15014	Zapopan, Jalisco	September 20 14.00–15.00 h	W 103° 19.95 N 20° 40.67	1,585	Seasonal–dry tropical forest secondary vegetation
CT	15421	Tuxtla Gutiérrez, Chiapas	September 28 11.00–14.00 h	W 93° 05.95 N 16° 49.52	559	Pine oak forest
QJ	15018	Jalpan de Serra, Querétaro	September 15 15.00–16.00 h	W 99° 28.57 N 21° 28.50	1,548	Pine-oak forest

Table 2

Accession numbers of sequences used in the phylogenetic study.

Accession numbers/species	Accessions numbers/species	Accessions numbers/species
<i>matK</i> AB233800 (<i>Galphimia glauca</i>) HQ247275 (<i>Galphimibrasiliensis</i>) AF344545 (<i>Galphimiagracilis</i>) HQ247277 (<i>Galphimiaspeciosa</i>) HQ247276 (<i>Galphimiamirandae</i>)	<i>rbcL</i> AB233904 (<i>Galphimia glauca</i>) HQ247487 (<i>Galphimibrasiliensis</i>) AF344475 (<i>Galphimiagracilis</i>) HQ247489 (<i>Galphimiaspeciosa</i>) HQ247488 (<i>Galphimiamulticaulis</i>)	<i>rpoC1</i> Sequences not available in GenBank
<i>matK</i> out group HQ247185 (<i>Aenigmatanthera lasiandra</i>) HQ247238 (<i>Byrsonima crista</i>) AB233794 (<i>Byrsonimacrassifolia</i>)	<i>rbcL</i> out group HQ247440 (<i>Banisteriopsiscaapi</i>) HQ247433 (<i>Amorimiarigida</i>)	<i>rpoC1</i> out group FJ038500 (<i>Terminaliaguyanensis</i>) FJ038833 (<i>Erismaucinatum</i>) FJ038768 (<i>Bunchosiasp.</i>) GQ429092 (<i>Byrsonimacrassifolia</i>)

Table 3

Accession numbers for the DNA sequences of all three DNA barcodes deposited in the Genbank.

Populations	Accession numbers (<i>matK</i>)	Accessions numbers (<i>rpoCI</i>)	Accessions numbers (<i>rbcL</i>)
GM	JX088054	JX125630	JX125620
QJ	JX088053	JX125628	JX125622
MM	JX088055	JX125627	JX125621
MT	JX088056	JX125626	JX125623
MC	JX088058	JX125625	JX125624
JG	JX088057	JX125629	JX125619
CT	JX088052	JX125631	JX125618