GENETIC DIVERSITY WITHIN AND AMONG WILD AND GARDEN AROMATIC SPECIES OF THE GENERA *Lippia, Aloysia* AND *Phyla* IN SEVERAL LOCATIONS IN NORTHEASTERN COLOMBIA

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Title: Genetic Genetic diversity within and among wild and garden aromatic species of the genera *Lippia, Aloysia* and *Phyla* in several locations in northeastern Colombia*

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Key words: *Lippia, Aloysia, Phyla, Lantana,* Verbenaceae, Genetic diversity

Species from the genera *Lippia, Lantana, Aloysia* and *Phyla,* have the potential to become commercial crops due to their essential oils. Nevertheless, the relationships among them have been poorly studied from the taxonomical and phylogenetical points of view. In this study, we document the levels of genetic diversity and population structure in *Lippia alba*, *L. origanoides, Phyla dulcis* and *Aloysia triphylla* from north-eastern Colombia, which differ in their mode of propagation (natural *versus* human-induced clonal propagation) and their environments (natural versus home gardens). We also studied the genetic relationships among these four closely related genera. For these purposes, two cpDNA regions, the internal transcribed spacer of the ribosomal DNA (ITS), and ISSR (Intersimple Sequence Repeats) markers were used. The results suggest that, in spite of being mainly clonally propagated, garden populations of *L. alba* and *A. triphylla* keep genetic diversity levels that are comparable to the naturally occurring population of *L. origanoides*, suggesting that garden populations may be important reservoirs of genetic diversity in these aromatic species. The analyses also suggest that the genera *Lippia*, *Phyla* and *Lantana* are more related to each other than they are to the genus *Aloysia*. Some *Lippia* species seem to be more closely related to other species from the genus *Lantana* than to species from the same genus. Further analyses are needed in order to address the relationships among these closely related genera and the monophyletic nature of them.

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Titulo: Diversidad genetica en y entre especies aromaticas, silvestres y de jardin, del genero *Lippia, Aloysia* y *Phyla* en diferentes localidades del nororiente Colombiano*

Autor: Felix Martinez Nuñez

Palabras Claves: *Lippia, Aloysia, Phyla, Lantana,* Verbenaceae, Diverdidad genetica

Especies del genero *Lippia, Lantana, Aloysia* y *Phyla* tienen el potencial de convertirse en importantes especies a nivel comercial, debido a su alto contenido de aceites esenciales. Sin embargo las relaciones entre estas no han sido muy bien estudiadas desde el punto de vista taxonómico y filogenético. En ese estudio, se documentraon los niveles de diversidad genética y estructura poblaciónal en *Lippia alba*, *L. origanoides, Phyla dulcis* y *Aloysia triphylla* del nororiente Colombiano, las cuales difieren en su modo de propagación (natural versus propagación clonal inducida por el hombre) y su distribución (ambiente natural versus jardines). Tambien se estudiaron las relaciones genéticas entre estos cutro generos relacionados. Para este propósito, se implementaron dos regiones no codificantes del cloroplasto, el espaciador transcrito interno del ADN ribosomal (ITS) y inter-microsatelites (ISSR) como marcadores moleculares. Estos resultados sugieren que, a pesar de que estas plantas sean propagadas clonalmente, las especies de jardín de *L. alba* y *A. triphylla* mantienen niveles similares de diversidad genética comparadas con la especie distribuida naturalmente *L. Origanoides*. Esto sugere que las poblaciones de jardín pueden ser importantes reservorios de diversidad genética en estas especies aromaticas. Este análisis también sugiere que los generos *Lippia*, *Phyla* y *Lantana* están más relacionadas entre ellas que con el género *Aloysia*. Algumas especies de *Lippia* parecen estar más relacionadas a otras especies del genero *Lantana* que con otras especies del mismo género. Mayores análisis son necesarios en orden de establecer las relaciones entre estos generos relacionados y su naturaleza monofiletica.

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1. INTRODUCTION:

The Verbenaceae family includes about 100 genera and 2,600 species of herbs, shrubs, and trees, mostly characterized for his aromatic nature. This family is a cosmopolita taxon, present on tropical, sub-tropical and temperate regions (Maish 1885). The Verbenaceae family has been treated in several ways in the literature. Different classification systems have been proposed for *Lippia* based on morphological characters (Moldenke 1956; Troncoso 1974; Troncoso 1979). Some authors consider it to be composed of three subfamilies: Verbenoideae, Vitioicoideae and Avicennoideae (Smith 1977). The limits of the Verbenaceae family are not well established and it ranges from a classification *sensu lato* (including 7 or 8 subfamilies) to a classificaction *sensu stricto* (including only one subfamily). Authors such as Briquet (1895, 1897) and Bethman (1876) recognized 7 and 8 subfamilies, respectively, within Verbenaceae s.l. (see Table 1 in Wagsttaf and Olmstead, 1997). Other authors recognize two tribes into family Verbenaceae s.str., where only two tribes are included. Tribe 745 contains the genera *Lippia, Lantana, Aloysia* and *Phyla,* and tribe 784 contains the genera *Bouchea, Stachytarpheta, Citharexylum, Duranta, Verbena,* and *Priva* (El-Gazzar and Watson 1970). Frequently, the genera *Lippia, Lantana, Phyla* and *Aloysia* are placed in the Tribe

Lantaneae or Lippieae, depending on the author (Caro 1982, Junell 1934, Méndez 1998, Romero *et al* 2002, Sanders 2001). In spite of the great potential of these four genera at the industry level, the relationships among them have been poorly studied from the taxonomical and phylogenetical points of view. Most of studies in this family have addressed relationships at higher hierarchical levels and most of them are based on anatomical and morphological observations, essential oils, seed oils and chromosome number (El-Gazzar and Watson 1970, Poser *et al*. 1997, Múlgura *et al*. 2002, Brandão *et al*. 2007). There are several taxonomical and phylogenetical problems between and within these genera, including various reports of synonymy, some of these are listed in Table 1. Currently, molecular taxonomic studies are carryout in order to clarify the *Lippia* and *Lantana* limits inside of what has been called the *Lantana*-*Lippia* complex (Lu-Irving and Olmstead, personal communication). Previous results from these studies suggest that the relationships between the species inside this complex will not be easy to resolve because of short branch lengths and also suggest that *Lippia* and *Lantana* may not be monophyletic genera (Lu-Irving and Olmstead personal communication).

Species from the genera *Aloysia, Phyla, Lippia* and *Lantana* are distributed from northern North America to South America; also there

are some reports on Asia and Africa (Botta 1979, Maisch 1885,). *Lantana camara*, *Lippia alba*, *Phyla nodiflora* and *Aloysia gratissima* are among the most widely distributed species for these genera and they occur in South America, Mesoamerica, the Caribbean and southern North America. In addition, *L. camara*, *L. alba* and *P. nodiflora* also occur in Africa and tropical Asia. Some species occur only in Mesoamerica and South America, among these are *P. dulcis* and *A. triphylla*. There are other species that have more restricted distributions, (such as *L. origanoides*, *L. glandulosa*, *L. filifolia*, *L. florida*, *L. rotundifolia*, *L. glandulosa*, *L. sidoides*, *L. lupulina*, *L. corymbosa* and *L. diamantinensis*, from South America, mostly from Brazil) and two species of the genus *Aloysia* (*A. wrightii* and *A. macrostachya*) which have reports from southern North America (Missouri Botanical Garden www.mobot.org, New York Botanical Garden www.nybg.org).

In Colombia, four species from the Verbenaceae family, namely *Lippia alba* (Mill) N.E. Br*., Aloysia triphylla* (L'Her.), *Phyla dulcis* Trevir and *L. origanoides* Kunth have potential economic value because of their essentials oils. These species are mostly used in pharmaceutics, food, textiles, fine organic chemistry, cosmetic and perfumery (Stashenko *et al*., 2003, Santos *et al* 2004, Cáceres *et al* 2006). *L. alba*, original from the Neotropics and populary known as "pronto alivio" ("which literally

means “quick relief”) or “bushy matgrass”, has been used in infusions as a tranquilizer, as well as for gastrointestinal disorders, presents sedative properties, antiulcerogenic activity, motor relaxant effects and provoke cardiac rate reduction (Arango 2004; Díaz 2003; Gazola *et al*. 2004; Vale *et al*. 2002; Pascual *et al*. 2001a; Pascual *et al*. 2001b; United-Nations 2005; Zetola *et al*., 2002). *A. triphylla* is characterized by its citric scent and its common name is “cidron”. Traditionally *A. triphylla* has been employed as a spice, digestive and sedative plant in beverages, also has antimicrobial activity (Carnat *et al* 1999, Muñoz *et al* 2001). *P. dulcis*, popularly known as “hierba dulce” or “hierba luisa”, has anti-inflammatory and immunomodulator activity (Cáceres *et al*. 2006). *L. origanoides*, also known as “oregano de monte” possesses antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Candida tropicali*s (Santos *et al*. 2004). Likewise, leaves of *L. origanoides* are used as a substitute of common marjoram (Maisch 1885).

Despite these well-known properties, very little is known about the basic aspects of the biology and genetics of these species. In Colombia, populations of *L. origanoides* occur naturally in semi-arid areas while the other three species are mainly found in home gardens where they are mainly clonally propagated by humans (Arango 2004 and personal

observations). Recent studies have suggested that home gardens may be important reservoirs of unique genetic diversity, which has led to initiatives to gain more understanding of the role of these "farming systems" in the management and conservation of genetic diversity *in situ* (Watson and Eyzaguirre 2002). Genetic diversity, and its distribution within and among populations, is affected by evolutionary forces (such as mutation, migration, genetic drift and selection), and by life history traits such as mode of reproduction (autogamous *versus* allogamous) and mode of propagation (by seeds or clonal). In this way, highly polymorphic genetic markers represent a powerful tool to infer levels of genetic diversity and genetic structure (Hardy *et al* 2005). In plants, their three genomes [mitochondrial DNA (mtDNA), chloroplast DNA (cpDNA) and nuclear DNA (nDNA)] have different modes of transmission (Birky 1995) and different rate of change (Wolfe *et al* 1987), and hence these can reveal different patterns of genetic variation (Petit *et al* 2005). In this context, the aim of this work is to document levels of genetic diversity and population structure in four economically important species of the Verbenaceae family from Colombia (*L. alba*, *L. origanoides*, *A. triphylla* and *P. dulcis*) with contrasting distributions (natural environments *versus* home gardens) and contrasting modes of propagation (natural *versus* human-induced clonal propagation). Also, by comparing the genetic data derived from this study and data freely

available from genetic databases, we look to study the genetic relationships among the closely related genera *Lippia, Lantana, Phyla* and *Aloysia*. For these purposes, we PCR-amplified and sequenced two cpDNA regions and the internal transcribed spacer of the ribosomal DNA (ITS), and also used ISSR (Intersimple Sequence Repeats) markers.

2. MATERIALS AND METHODS

2.1. Sample Collection

A total of 42 individuals from the species *L. origanoides*, *L. alba*, *P. dulcis* and *A. triphylla* were collected at 13 sites in the northeastern Andes of Colombia (departments of Norte de Santander, Santander and Cundinamarca) (Figure 1, Table 2). For each collection, complete passport data were recorded and herbarium samples were deposited at the Colombian National Herbarium (COL). *L. origanoides* was collected from a natural population at the Chicamocha canyon in the department of Santander, specifically in the localities of Pescadero and Cepitá. This is a low-dry area (about 500 altitude meters) and markedly seasonal. Although the reproductive mode of this species has not been studied,

the presence of odoriferous compounds suggests that pollinators may visit these plants, which would encourage open pollination among different individuals. Plants of *L. alba*, *P. dulcis* and *A. triphylla* were collected in home gardens, were peasants propagate them clonally by means of stem cuttings.

2.2. DNA extraction, PCR amplification and sequencing

One to nine individuals were analyzed per each collection site. From each individual, 20 to 30 young leaves were taken, preserved with silica gel in zip-lock plastic bags and stored at -20ºC until total genomic DNA extraction. Leaf samples were homogenized to fine powder in liquid nitrogen and DNA was extracted following the CTAB method modified by Pirttilä *et al* (2001).

A set of 50 ISSR primers (obtained from the University of British Columbia in Vancouver, Canada) were screened for polymorphisms and amplification success in a representative sample of *L. origanoides*, *L. alba*, *P. dulcis* and *A. triphylla* in order to identify suitable markers for population genetic analyses. PCR reactions contained 1 Unit of *Taq* polymerase (Promega), 1X PCR Buffer (Promega), 0.2mM dNTPs, 0.5μM of the ISSR primer and about 10-20 ng of extracted DNA.

Amplifications were performed on a PTC-100^{TM} thermocycler (MJ Reserch, Inc) following Wolfe (2005) cycling conditions. PCR products were loaded onto 2% agarose gels in 0.5X TBE buffer, run at constant voltage (50 V) for 3 h and detected by staining with ethidium bromide. Each ISSR band was considered as an independent character or locus, and polymorphic bands were scored visually as biallelic states, either absent (0) or present (1). Qualitative differences in band intensity were not considered (Assefa *et al* 2003). Two independent scorings were made on each gel, and only those bands consistently scored were considered for analysis.

Two non-coding cpDNA regions (the intergenic spacers *rps*14–*psa*B and *trn*L–*trn*F) and the ITS region (ITS1 and ITS2) were amplified in the 42 individuals. PCR reactions contained 1X PCR Buffer (Promega), 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM of each primer (primers ITS1 and ITS4 reported by White *et al*. 1990), 1 Unit of *Taq* poymerase (Promega) and about 10-20 ng of DNA. Amplifications were performed on a PTC-100^{TM} thermocycler (MJ Reserch, Inc) under the following conditions: initial denaturation at 94ºC for 4 min; followed by 1 min at 94ºC, 1min at 50ºC, and 2 min at 72ºC for 40 cycles, and 10 min at 72ºC for a final extension. PCR products were loaded on 1% agarose gels and detected by staining with ethidium bromide. The best PCR products

were sent to Macrogen (www.macrogen.com) to be sequenced. All sequences were submitted to GenBank under the accession numbers

To study the genetic relationships among the closely related genera *Lippia, Lantana, Phyla* and *Aloysia*, sequences from the ITS1 region of several *Lippia* species from Brazil and other Verbenaceae species were obtained from GenBank (Table 3).

2.3. Data analysis:

2.3.1. Genetic diversity and population genetic analyses in wild and garden species from Colombia

The ISSR polymorphisms were described as the percentage of polymorphic loci (P) and the intraspecific genetic diversity was estimated by the Shannon's diversity index (Shannon and Weaver 1949) by assuming Hardy-Weinberg equilibrium (HWE) as implemented in POPGENE v. 1.31 (Yeh *et al* 1995) and by using a Bayesian approach which does not assume HWE as implemented in the software HICKORY (Holsinger *et al* 2002). Population structure for the species *L. alba, L. citriodora* and *L. origanoides* was studied by means of clustering and multivariate methods, without assuming populations *a priori*. A similarity matrix among individuals was produced based on the similarity coefficient of Nei and Li (1979). This matrix was used to construct a

dendrogram based on the UPGMA algorithm. Also, a principal coordinate analysis (PCO) was carried out with the Multivariate Statistical Package MVSP (Kovach 1993). An estimation of inter-population differentiation (θ^B) for *L. alba* was carried out by applying the Bayesian approach with HICKORY (Holsinger *et al* 2002). The Bayesian method allows direct estimates of F_{ST} from dominant markers (Holsinger *et al* 2002).

Sequences of the two cpDNA regions (*trn*L-*trn*F and *rps*14-*psa*B) were edited and aligned using Muscle V. 3.6 (Robert 2004), followed by manual adjustment. Nucleotide diversity was measured as the number of polymorphic sites (P) and the nucleotide diversity using the software DNAsp V. 3.2 (Rozas and Rozas 1995). A combined alignment of the two cpDNA regions was generated and a genetic distance matrix among individuals was estimated using different models of nucleotide substitution: F84 (Felsenstein and Churchill 1996), Jukes and Cantor (1969) and Kimura-2 parameters (Kimura, 1980). The distance matrixes were used to build a dendrogram by applying the NJ algorithm (Saitou and Nei 1987). Support for groups in the dendrogram was assessed through bootstrap analyses (1000 replicates) with the software SeqBoot from the PHYLIP package (Felsenstein 1995).

2.3.2. Genetic relationships among *Lippia* species from Colombia, Brazil and other genera

In order to assess the genetic relationships among *Lippia* species from Colombia and Brazil, and among the genera *Lippia*, *Lantana*, *Aloysia* and *Phyla*, ITS sequences were either empirically obtained or accessed through the GenBank database (Table 3). As described above, sequences were aligned using Muscle V. 3.6 (Robert 2004) and distance matrixes were generated under different models of nucleotide substitution. Phylogenetic analyses were carried out under the maximum parsimony (MP) criterion (Fitch 1971) and by using the methods of Fitch–Margoliash (Fitch and Margoliash 1967) and Neighbour Joining (NJ) as implemented in the PHYLIP package (Felsenstein 1995). Support for phylogenetic groups was assessed through bootstrap analyses (1000 replicates) with the software SeqBoot from the PHYLIP package (Felsenstein 1995). The genus *Verbena* was selected as outgroup on the basis of previous morphological works (Moldenke 1956; Troncoso 1974; Troncoso 1979) and the molecular phylogenetic studies of Olmstead *et al* on the *Lantana-Lippia* and the Verbena complexes (Olmstead, personal communication).

3. RESULTS

3.1. Genetic diversity and population genetic analyses in wild and garden species from Colombia

A total of five ISSR primers were chosen due to their ability to produce polymorphic and interpretable bands among the species *L. alba, L. origanoides* and *A. triphylla* from Colombia. The five primers used are the following: #813 (CTC TCT CTC TCT CTC TT), #814 (CTC TCT CTC TCT CTC TA), #824 (TCT CTC TCT CTC TCT CG), #843 (CTC TCT CTC TCT CTC TRA) and #845 (CTC TCT CTC TCT CTC TRG). These five primers produced a total of 88 scorable bands, of which 75 were polymorphic for *L. alba* (85.23%), 84 for *A. triphylla* (95.45%) and 76 for *L. origanoides* (86.21%) (Table 4). *P. dulcis* was excluded from this analysis due to the small number of samples. Due to the dominant nature of the ISSR markers, some analyses could produce biased results since HWE is often assumed to estimate the allele frequencies. For this reason, a Bayesian analysis was performed to estimate genetic diversity. The Bayesian analysis showed slightly higher levels of genetic diversity compared to the Shannon index (Table 4). The levels of genetic diversity among the wild species (*L. origanoides*) and the home garden species (*L. alba* and *A. triphylla*) are very similar, which suggests

these garden species may be a reservoir of useful genetic diversity (Tabla 4).

A total of 710 pb for the two intergenic spacers (*rps*14-*psa*B and *trn*L-*trn*F) in 41 individuals was analyzed. The nucleotide composition for these two regions was of 60.9% A-T and 39.1 % G-C. Among the four species, 681 monomorphic sites (95.9 %) and 29 polymorphic sites (4.1 %) were observed (Table 5). Twenty-six out of the 29 polymorphic sites were parsimony informative, and 3 were singleton variable sites. The region *trn*L-*trn*F showed the higher number of polymorphic sites (Table 5). In this region, 22 polymorphic sites were observed; 1 as a singleton variable site and 21 were parsimony informative. In the region *rps*14-*psa*B only 7 polymorphisms were observed and 2 of them were singletons. Average nucleotide diversity (Pi) for all the 4 species was 0.0037 for *rps*14-*psa*B and 0.0247 for *trn*L-*trn*F, with an average of 0.0142 for the two regions. Only in *L. alba* interspesific polymorphism was detected (Table 5).

The cluster and PCO analyses of ISSR data and the NJ analysis of cpDNA data allowed assessing the population structure among and within the four species, each of which clustered into discrete groups

(Figures 2, 3 and 4). The similarity matrix among populations based on the Nei and Li coefficient is shown in Appendix 1. The cluster, PCO and NJ analyses showed the possible existence of two different geographical clusters within *L. alba*: Norte de Santander and Santander. The level of differentiation between the populations of *L. alba* from these two regions was relatively low but significant as measured by the Bayesian approach implemented in the software hickory (θ^B=0.03, s.d=0.29, range=0.012-0.054). The only one individual of *A. triphylla* from the department of Cundinamarca (UIS 23-1) included in the study clustered apart from the other individuals of *A. triphylla* from the departments of Santander and Norte de Santander (Figures 2 and 3). Due to the small sample size for *P. dulcis*, there is not a clear clustering pattern among them.

3.2. Genetic relationships among *Lippia* species from Colombia, Brazil and other genera

The genetic relationships among species were studied by different approaches: the MP method, the Fitch-Margoliash algorithm and the NJ algorithm. However, only results from NJ analyses are shown because the MP method produced topologies not well-resolved and groups in the Fitch-Margoliash topologies did not get good bootstrap support.

In order to examine the genetic relationships among *Lippia* species from Colombia and Brazil, and among the closely genera *Lippia*, *Phyla*, *Aloysia* and *Lantana*, ITS1 sequences generated in this study and freely available from public databases were used (Table 3). The NJ dendrograms based on different models of nucleotide substitution gave similar results (data nor shown). The results suggest that the genus *Lippia*, *Phyla* and *Lantana* are more related to each other than they are to the genus *Aloysia* (Figure 5). It can also be observed that some *Lippia* species seem to be more closely related to species from the genus *Lantana* than other species from the same genus. Although the sample size analyzed is too small to make conclusions, this result agrees with previous unpublished data where *Lippia* and *Lantana* do not seem to be monophyletic genera (Lu-Irving and Olmstead personal communication).

4. DISCUSSION

4.1. Genetic diversity and population structure in wild and garden species from Colombia

This is the first analysis on the genetic diversity and population structure in wild and garden species from the Verbenaceae family (*L. alba, A.*

triphylla, Phyla dulcis and *L. origanoides*) in Colombia by means of chloroplast (*rps*14-*psa*B and *trn*L-*trn*F) and nuclear (ISSRs) markers. These results bring three points for discussion: (1) the relatively similar levels of genetic diversity found in wild and garden species in Colombia and the role of home gardens as reservoirs of genetic diversity, (2) the geographical structuring of the genetic diversity in *L. alba* and the consequences for its utilization and conservation, and (3) the utility of ISSR markers in genotypic identification in species from the genera *Lippia*, *Phyla* and *Aloysia* and their future use for propagation and breeding programs.

4.1.1. Home gardens as reservoirs of genetic diversity

The ISSR analyses showed relatively high levels of genetic diversity in *L. alba, A. triphylla,* and *L. origanoides*. The genetic diversity levels found here are similar or slightly higher than those obtained in previous studies in another aromatic and medicinal Verbenaceae species. For example, in *Vitex negundo* L. populations from China, similar percentages (ranging from 80.38%-85.47%) or lower percentages (ranging from 65.8%-82.9%) of polymorphic loci and also lower values of genetic diversity (ranging from 0.20-0.30) based on RAPD polymorphisms were observed (Su *et al* 2003, Zhang *et al* 2007). The

relatively high levels of genetic diversity found in this study (Ht=0.436-0.484) are similar to the levels found in other outcrossing species in other plant families (Broyles *et al* 1997, Fu and Dane 2003, Uesugi *et al* 2004, Apte *et al* 2006). Previous studies suggest that the breeding system have significant influences on genetic diversity in populations (Hamrick and Godt 1996). Costich and Meaghers (1992) suggested that outcrossing species should maintain higher levels of genetic variation than selfing species within populations. In this way it could be suggested that the garden species (*L. alba* and *A. triphylla*) analyzed in this study, which are mainly clonally propagated by cuttings, maintain levels of diversity similar to wild populations (*L. origanoides*) and in general, to species with outcrossing mating systems. These results suggest that home gardens may offer a kind of microenvironments where high levels of genetic diversity within larger farming systems may be contained. These gardens are not only important sources of food, medicine and other useful products for human but also may be important for *in situ* conservation of a wide range of plant genetic resources. Often the strong influence of human, managing the gardens leads to increased diversity converting home gardens in important centers of experimentation, plant introduction, and crop improvement as well as refuges for unique genetic diversity (Watson and Eyzaguirre 2002). In this way, plants in gardens have the potential to contribute to

increase genetic diversity, effective size of populations, and levels of genetic connectedness (McRoberts *et al* 2005). In summary, the complex species diversity and interactions in home gardens could be important systems for the study of evolution of plant genetic resources (Watson and Eyzaguirre 2002).

4.1.2. Population structure within *L. alba*

In this study, population differentiation on the basis of geographic location was found for *L. alba* from the departments of Norte de Santander and Santander. This geographic pattern of genetic variation was clearly visualized in the UPGMA dendogram and the PCO plot. Additionally, the Bayesian analysis showed a low but significant level of differentiation between the samples from the two geographic locations. These results may reflect a recent population diversification event in these two regions and a low level of interchange of genotypes from one location to the other, which could promote or keep genetic differentiation. *L. alba* occurs widely within Colombia and at different elevations. The present results will undoubtedly encourage further studies in this species in order to understand the source of the genetic variation and to provide useful information for breeding and conservation programs of this promising species.

4.1.3. Utility of ISSR markers for genotype identification

These results have demonstrated that ISSR markers can be used in genetic diversity studies as well as in genotypic identification in *Lippia, Aloysia* and *Phyla* as it has been confirmed for other species (Debnath 2007, Han *et al.* 2007, Aga *et al*. 2005, Weiguo *et al*. 2007, Culley and Wolfe 2001). In this study, ISSR markers showed to be highly polymorphic and able to distinguish among individuals, which make them potentially useful markers for cultivar identification and for solving problems in germplasm management and nursery mislabeling (Lopes *et al* 2007).

The genera *Lippia*, *Phyla* and *Aloysia* are still largely unexplored and these kinds of markers provide an opportunity to document the genetic resources available in these genera which is especially important for future programs that include their domestication. So far, we know that different types of chemotypes have been reported for these genera. For *L. alba* three chemotypes have been identified, which are probably due to genotypic variations (Tavares *et al*. 2004). In this study, different genotype groups were found in Santander and Norte de Santander and further analyses of essential oil composition are needed to confirm the

presence of different chemotypes among these two localities. *L. origanoides* has shown variation in the carvacol concentration (Santos *et al* 2004), and there is one report on the existence of at least two different chemotypes of this species in different locations in Colombia (Jarvis *et al* 2006). In this way, further analyses of different populations of *L. origanoides* in Colombia are needed in order to correlate genetic diversity and variation in essential oil composition.

4.2. Genetic relationships among *Lippia* species from Colombia, Brazil and other genera

The genetic relationships among *Lippia* species from Colombia and Brazil and among the closely related genera *Lippia*, *Aloysia*, *Lantana* and *Phyla* were studied by means of ITS markers and based on genetic distance and parsimony analyses. The present results bring two main points for discussion, (1) the genetic relationships among *Lippia* species from Colombia and Brazil and the current taxonomical classification of the genus *Lippia* in different sections, and (2) the closer relationship among the genera *Lippia*, *Phyla* and *Lantana* than among these genera and the genus *Aloysia*.

Different classification systems have been proposed for *Lippia* based on morphological characters (Moldenke 1956; Troncoso 1974; Troncoso 1979). Our analyses included species from four generally accepted sections, whose ITS1 sequences are freely available from databases or were obtained in this study (Figure 5). From Section Zapania: *L. corymbosa, L. diamantinensis, L. hermannioides* and *L. rubella*, all of them from Brazil, and *L. alba* from Colombia and Brazil. From Section Rhodolippia: *L. florida, L. lupulina, L. pseudothea* and *L. rosella*, all of them from Brazil. From section Goniostlachyum: *L. glandulosa* and *L. sidoides*, both from Brazil, and from section Dioicolippia: *L. filifolia* from Brazil.

The groups obtained according to the NJ tree shown in Figure 5 indicate that some species that are classified in different sections on the basis of morphological features occur in the same ITS1 cluster. For example, the species *L. florida* and *L. filifolia* from the sections Rhodolippia and Dioicolippia, respectively, clustered together with 89% bootstrap support. On the other hand, some groupings include species from the same section, for example *L. corymbosa* and *L. diamantinensis* from section Zapania are included in the same clade, and also, *L. glandulosa* and *L. sidoides* from section Goniotlachyum clustered together. Although this analysis does not include all the species from all the sections within *Lippia*, this preliminary result suggests that the

classification based on morphological characters may conflict with the one obtained from molecular data, especially for Section Zapania (Figure 5). This last section has been previously reported as a complex section inside the genus *Lippia* (Viccini et al 2005). Viccini *et al* (2004) based on RAPD data proposed that the species from section Rhodolippia form a relatively closely related group. However, in our results, the species from section Rhodolippia did not form a single related cluster (Figure 5). These results suggest that molecular phylogenetic analysis are important, to contribute in the taxonomical classification within *Lippia*.

The results also revealed that the genera *Lippia*, *Lantana* and *Phyla* are more related to each other than they are to *Aloysia* (Figure 5). The close relationship between the genera *Lippia* and *Lantana* was evident in this study supporting the results from previous cytogenetic and chemical analyses. In cytogenetic analyses, *Lippia alba* and *Lantana camara* shared common cytogenetic characteristics, such as the presence of a large block of heterochromatin and the same number of rDNA sites (Brandão *et al*. 2007). In iridoid and phenylethanoid glycosides analyses, the isolation of compound 3 (lamiide-type) in *Lantana camara* and *Lippia alba* corroborates the close relationship of both genera (Taoubi *et al*., 1997, Barbosa *et al*., 2006). Analyses of iridoid glucoside

substitution patterns in the Verbenaceae family put the genus *Aloysia* as the ancient group among the four genera, from the chemical viewpoint, due to the ability to introduce an hydroxyl group in position 5 (Poser *et al*., 1997). This assumption might be supported in the NJ tree of the ITS1 region where the samples of *A. triphylla* grouped in a different clade separated from *Lippia* and *Phyla* (Figure 5). Despite the low number of samples analyzed for the ITS1 region, this region was capable to determine the relationships among the 4 genera (*Lippia, Lantana, Phyla* and *Aloysia*) and this region should be useful for future phylogenetic analyses.

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6. TABLES AND FIGURES

Table 1. Some cases of synonymy reported for the genera *Lippia*, *Lantana*, *Aloysia*, *Phyla* and *Verbena*.

Species	**Synonymy**	**Reference**
Lippia alba Mill (1925)	*Lippia geminata, K*unt (1825) *Lantana alba* Mill (1768).	Troncoso (1979)
Phyla dulcis Trevir, (1826)	*Lippia dulcis* Trevir (1826)	Stevens *et al.* (2001)
Lippia origanoides Kunth, (1817)	*Lippia schomburqkiana* Schauer(1847)	Boggan *et al* (1997)
Lippia rubella (Moldenke) T.R.S.Silva	*Lantana rubella* Moldenke (1940)	Silva and Salimena (2002)
Lippia lupulina Cham (1832)	*Lippia bradeana* Moldenke 1975	Salimena (2002)
Lantana camara L. (1753)	*Lantana aculeata* L. (1753)	Brako and Zarucchi (1993)
Phyla nodiflora (L.) Greene	*Lippia nodiflora* (L.) Greene	Moldenke (1956)
Aloysia triphylla, L'Hér (1925)	*Lippia citriodora* Kunth (1818)	Troncoso (1979)
Verbena officinalis	*Verbena setosa* M. Martens and Galeotti	Zuloaga (1997)

Table 2. Passport data of samples used in this study. N means number of individuals collected.

Collection Number	**Species (COL accession number)**	**Habitat**	**Municipality**	**N**
Santander				
UIS02	*P. dulcis* (512102)	Garden	Jordán Sube	2
UIS06	*L. alba* (512107)	Garden	San Gil	1
UIS11	*L. origanoides* (522847)	Wild	Pescadero/Cepitá	9
UIS12	*L. alba* (512082)	Garden	Bucaramanga	4
UIS16	*A. triphylla* (522839)	Garden	Floridablanca	3
UIS24	*L. alba* (512084)	Garden	Floridablanca	9
UIS26	*L. alba* (522844)	Garden	Bucaramanga	2
UIS27	*L. alba* (522851)	Garden	Bucaramanga	1
UIS28	*L. alba* (522852)	Garden	Bucaramanga	1
Norte de Santander				
UIS31	*A. triphylla*	Garden	Silos	1
UIS35	*A. triphylla*	Garden	Mutiscua	1
UIS41	*L. alba*	Garden	Cucuta	7
Cundinamarca				
UIS23	*A. triphylla*	Garden	Zipaquirá	1
Total				42

Table 3. List of the ITS1 sequences from GenBank analyzed in this study

GenBank accession No.	Species	Origin
AY945826	*Lippia sidoides*	Brazil
AY945825	*Lippia rubella*	Brazil
AY945823	*Lippia rosella*	Brazil
AY945822	*Lippia pseudothea*	Brazil
AY945821	*Lippia lupulina*	Brazil
AY945820	*Lippia hermannioides*	Brazil
AY945819	*Lippia glandulosa*	Brazil
AY945818	*Lippia florida*	Brazil
AY945817	*Lippia filifolia*	Brazil
AY945816	*Lippia diamantinensis*	Brazil
AY945815	*Lippia corymbosa*	Brazil
AY945827	*Lippia alba*	Brazil
AY945814	*Lippia alba*	Brazil
AF477784	*Lantana camara*	China
AF225294	*Lantana camara*	Brazil
AF437873	*Lantana camara*	Taiwan
AF437870	*Lantana camara*	Taiwan
DQ463783	*Lantana horrida*	USA
DQ463782	*Aloysia gratissima*	USA
AY928530	*Aloysia macrostachya*	USA
AY178652	*Aloysia macrostachya*	USA
AY928529	*Aloysia wrightii*	USA
AY178653	*Aloysia wrightii*	USA
AY928528	*Verbena halei*	USA
AY928527	*Verbena rigida*	USA
AY904022	*Phyla nodiflora*	USA
AY178654	*Phyla nodiflora*	USA

Table 4. Genetic diversity in wild and garden populations based on ISSR markers

Species	Habitat	P	SI	S.D.I.	Ht	S.D.Ht
Lippia origanoides	Wild	86.21%	0.453	0.274	0.484	0.0098
Lippia alba	Garden	85.23%	0.418	0.2493	0.436	0.0028
Aloysia triphylla	Garden	95.45%	0.436	0.1837	0.461	0.0069

P: percentage of polymorphic loci
SI: Shannon index
S.D.I: Standard deviation for the Shannon Index
Ht: Bayesian Genetic Diversity
S.D.Ht: Standard deviation for the Bayesian Genetic Diversity

Table 5. Genetic diversity in wild and garden populations based on two chloroplast regions.

cpDNA region	n	L	P	S	I	Habitat			
						Wild	Garden		
						Pi(LO)	Pi(LA)	Pi(AT)	Pi(PD)
*rps*14-*psa*B	41	459	7	2	5	0	0,00036	0	0
*trn*L-*trn*F	41	251	22	1	21	0	0,00068	0	0
Total/Mean	41	710	29	3	26	0	0.0005	0	0

n=number of sequences analyzed for the three species
L=Number of base pairs analyzed
P=Number of polymorphic sites.
S=singletons variable sites
I=Number of parsimony informative sites.
Pi(LO)= intraspecific nucleotide diversity for *L. origanoides*
Pi(LA)= intraspecific nucleotide diversity for *L. alba*
Pi(PD)= intraspecific nucleotide diversity for *P. dulcis*
Pi(AT)= intraspecific nucleotide diversity for *A. triphylla*

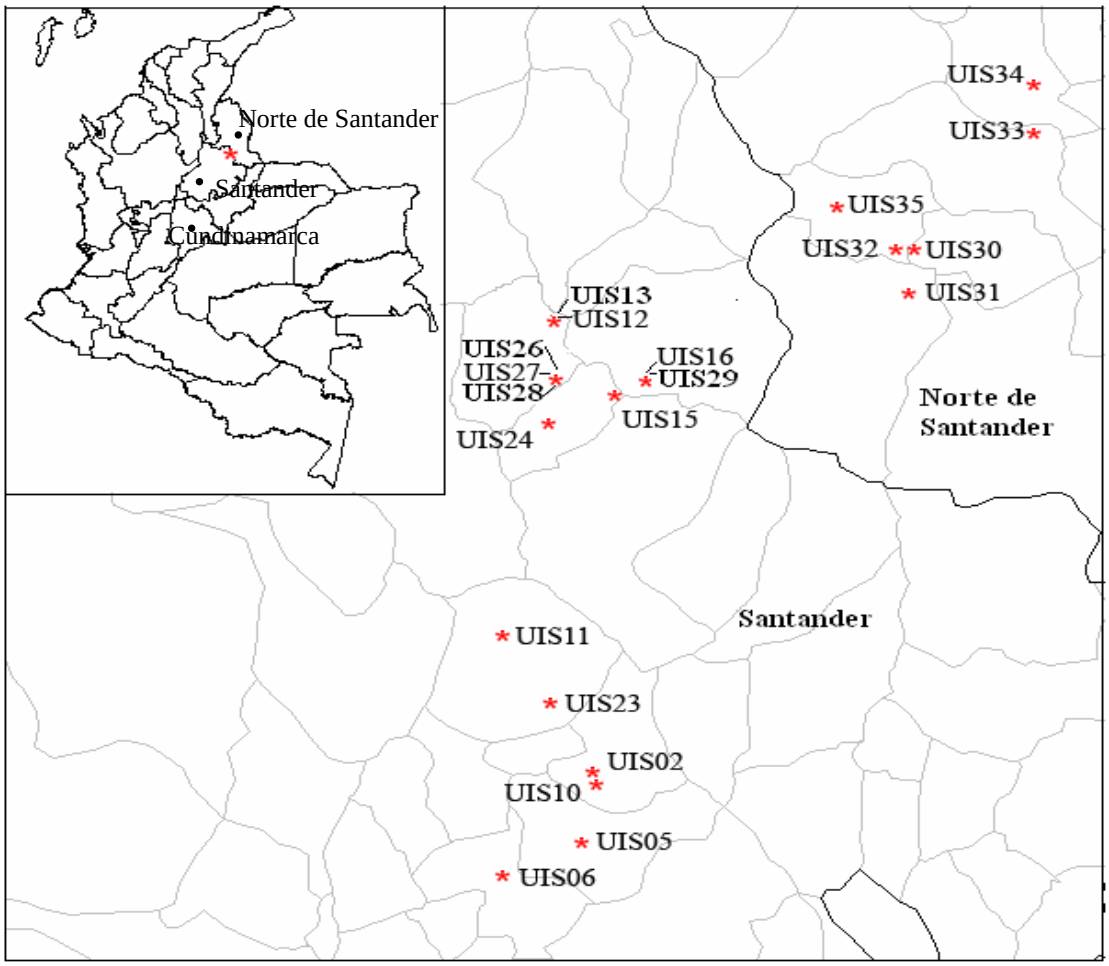

Figure 1. Collection sites of aromatics species used in this study. Asterisks indicate geographical sites and the UIS number indicates the accession number of the collection (see table 1)

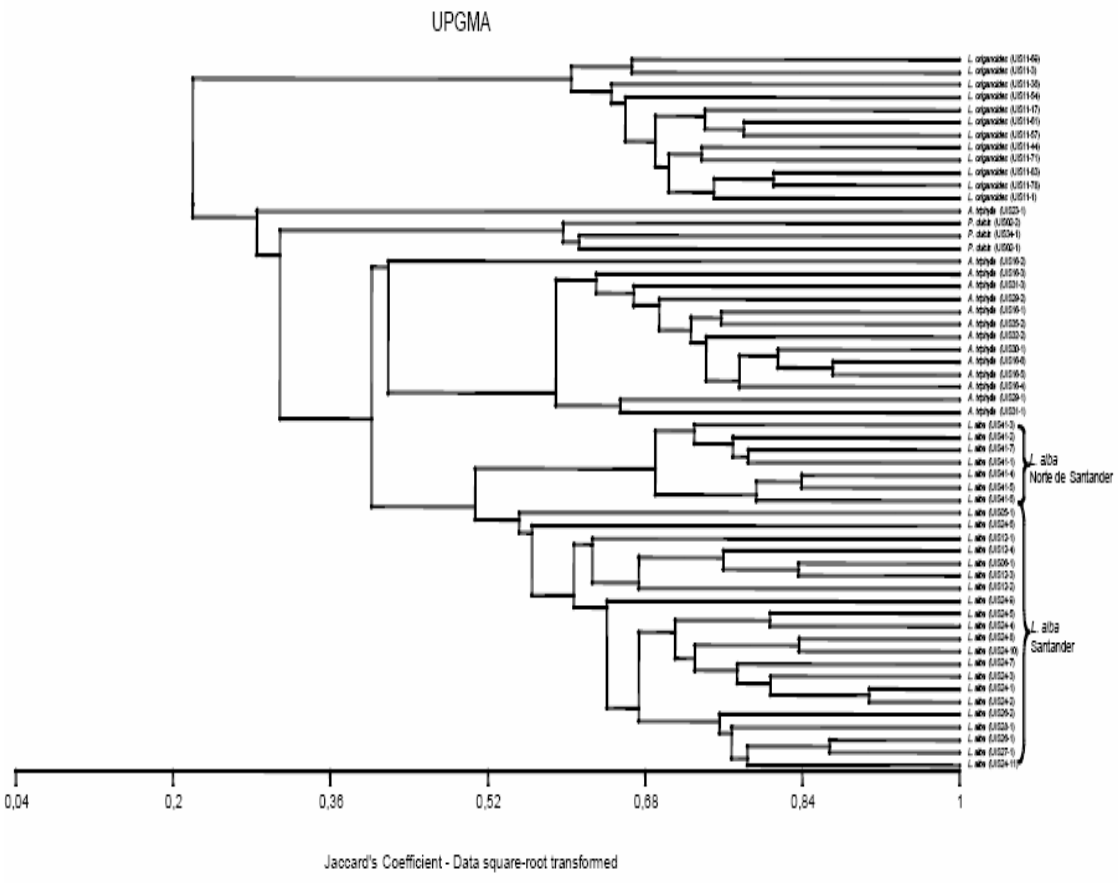

Figure 2. UPGMA dendrogram based on ISSR polymorphisms showing the genetic relationships among the wild and garden aromatic species used in this study.

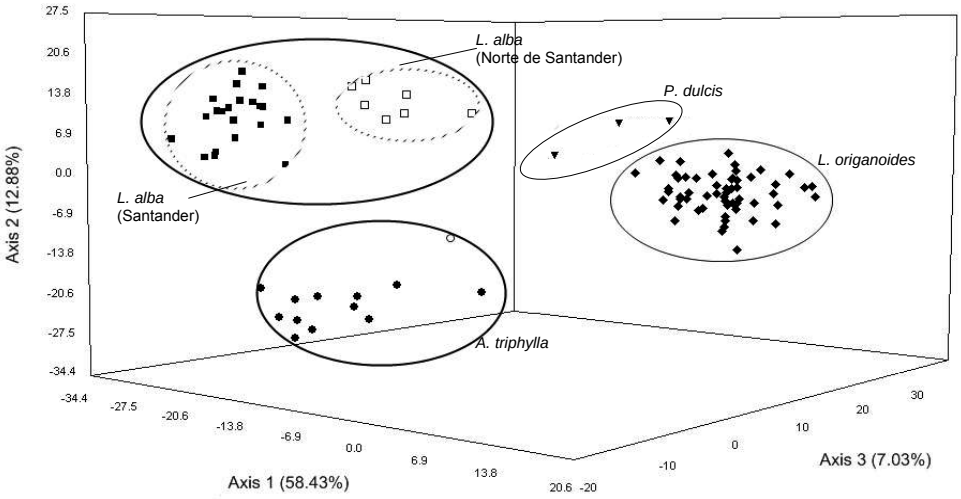

Figure 3. Principal Coordinates (PCO) analysis based on ISSR data and the Nei & Li genetic distance. The first two coordinates explain 71.31% of the total variability.

Figure 4. NJ dendrogram based on *trn*L-*trn*F and *rps*14-*psa*B sequences and Kimura 2-parameter model of nucleotide substitution showing the genetic relationships among the wild and garden aromatic species from Colombia used in this study. Numbers on the topology indicate bootstrap value based on 1000 replicates.

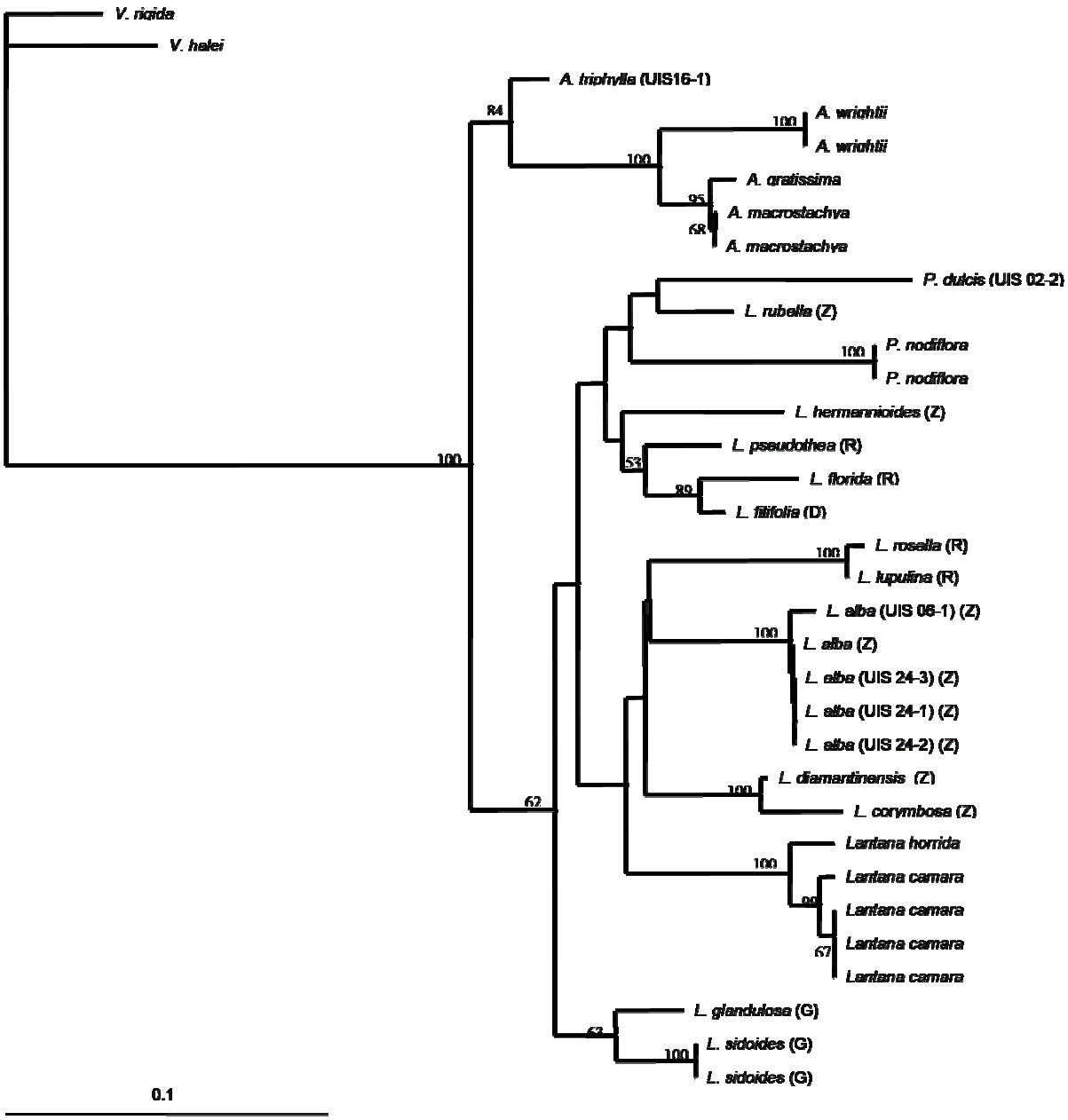

Figure 5. NJ dendrogram based on ITS1 sequences and Jukes-Cantor model of nucleotide substitution showing the genetic relationships among the genera *Lippia*, *Phyla*, *Lantana* and *Aloysia*. Numbers on the topology indicate bootstrap value based on 1000 replicates. (Z) Section Zapania, (R) Section Rhodolippia, (G) Section Goniostlachyum, (D) Section Dioicolippia.

7. APPENDIX 1.

Nei`s genetic distance matrix among the five species analyzed

	L. origanoides	***L. alba S.***	***L. alba N. S.***	***A. triphylla***	***P. dulcis***
L. origanoides	-	0.4501	0.5164	0.4410	0.5099
L. alba S.	0.4501	-	0.1810	0.2456	0.4114
L. alba N. S.	0.5164	0.1810	-	0.3321	0.3867
A. triphylla	0.4410	0.2456	0.3321	-	0.4621
P. dulcis	0.5099	0.4114	0.3867	0.4621	-

S = Species from Santander

NS = Species from Norte de Santander

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