A Preliminary Phylogeny of New World Verbenaceae Based on nrDNA sequences

Jorge Ruiz
Mentor:
Dr. Freeman
Abstract

This study of the New World Verbenoideae concerned evolutionary relationships of genera within the Verbenaceae (subfamily Verbenoideae) based on nuclear ribosomal Internal Transcribed Spacer (ITS) DNA sequences. The placement of many Verbenacean genera into families have been problematic with many traditional genera being segregated recently into the Lamiaceae. This study included only taxa of the Verbenaceae s. str. The data to date indicate three distinct clades within Verbenaceae. The first includes the genera Aloysia, Lantana, Lippia, Phyla, and Priva. The second includes Glandularia, Junellia, Stylodon, and Verbena. The third includes Bouchea and Stachytarpheta. Numerous genera remain to be sampled before a more complete picture of phylogeny in New World Verbenaceans can be hypothesized.

Introduction

The Order Lamiales is a controversial assemblage of many large families. Some of the smaller families are Avicenniaceae (Black Mangrove family), Buddlejaceae (Sagewood family), Martyniaceae (Devil’s claw family), Myoporaceae (Myoporum family), Oleaceae (Olive family), Pedaliaceae (Sesame family), Plantaginaceae (Plantain family) and the larger families in this Order are Acanthaceae (Acanthus family) 240 genera, 2200 spp., Boraginaceae (Borage family) 100 genera, 2000 spp., Bignoniaceae (Bignon family) 100 genera, 800 spp., Gesneriaceae (African violet family) 85 genera, 1200 spp., Lamiaceae (Mint family) 160 genera, 3500 spp., Scrophulariaceae (Snapdragon family) 200 genera, 2600 spp., and the Verbenaceae (Vervain or Lantana family) (Lawrence, 1951; Porter, 1967; Smith, 1977).
The traditional family Verbenaceae is a cosmopolitan, pantropical taxon with a few genera which reach warm temperate regions, and even the cool temperate regions (Lawrence, 1951). In this view, the family is composed of about 90 genera and 3000 species (Porter, 1967). They are herbs, shrubs, or trees. The flowers are bisexual, typically actinomorphic to slightly zygomorphic, small to medium-sized, 5-merous (occasionally 4-merous or 8-merous), and their stems are often four-sided (Lawrence, 1951; Smith 1977). Other characteristics of the Verbenaceae are that they have either anatopous ovules (micropyle close to funiculus attachment) or orthotropous ovules (erect and straight) (Smith 1977).

That the Lamiaceae and Verbenaceae are closely related has long been understood (Cronquist 1981). The families share numerous characters including opposite leaves, zygomorphic corollas, and a bicarpellate ovary that is divided into four locules (Wagstaff and Olmstead 1997). Both families usually have aromatic leaves. The traditional difference between the two families is the ovary. The Lamiaceae generally have a deeply four-lobed ovary with gynobasic style while the Verbenaceae have an unlobed ovary and a terminal style. It has long been understood that taxa with intermediate ovaries can be found in both families (Cronquist 1981). Thus, the circumscription of both families has been problematic.

Because of these difficulties, Cantino (1992) proposed a very different classification of these families from the previous literature (Bentham 1876; Briquet 1895 - 1897) but similar to that of Junell (1934). Thus, numerous genera previously considered a part of the Verbenaceae were transferred to the Lamiaceae, leaving a narrowly circumscribed Verbenaceae consisting of the subfamily Verbenoideae minus the tribe
Monochileae (Cantino 1992). These changes were accepted by Thorne (1992) who also segregated Avicenniaceae, Nesogenaceae, Phrymaceae, Stilbaceae, and Symphoremataceae. This narrower view of the taxon Verbenaceae is, then, Verbenaceae *sensu strictu*. The North American genera *Callicarpa* and *Tetraclea*, traditionally included within the Verbenaceae, have been recently removed to the Lamiaceae. Other problems are seen at higher taxonomic levels such as the validity of the Order Scrophulariales (e.g., Thorne 1992; Wagstaff and Olmstead 1997). These relationships are currently being investigated using molecular data.

The family Verbenaceae has been treated several ways in the literature. Some authorities (e.g. Smith 1977) consider it to be composed of three subfamilies. The first is the Verbenoideae with racemose or spicate inflorescences. This subfamily is made up of the genera *Verbena*, *Glandularia*, *Lantana*, *Phyla*, *Stylodon*, *Priva*, and *Duranta*. The second subfamily is the Vitioicoideae, which is distinguished by its cymose inflorescences. This subfamily is composed of the genera *Callicarpa*, *Tectona*, *Clerodendrum*, and *Vitex*. The third subfamily, Avicennoideae, is composed of the genus *Avicennia* (the black mangroves of coastal Louisiana to Florida in the United States). Many authorities (e.g. Moldenke et. al. 1936, 1946; Schwartzbach and McDade 2002) have segregated the genus *Avicennia* into the family Avicenniaceae. The genera of subfamily Vitioicoidae (e.g., *Callicarpa*, *Clerodendrum*, and *Vitex*) have been placed in the Lamiaceae based on DNA sequence data.

There is no published literature to our knowledge, however, concerning phylogenetic relationships within the New World Verbenaceaen s. str. This investigation is a beginning toward such a reconstruction.
We are trying to determine the evolutionary relationships of genera within the Verbenaceae (subfamily Verbenoideae) based on nucleic acid sequence data. In this study we used non-coding DNA sequences which presumably accumulate nucleic acid base changes faster than coding sequences. We sequenced the ITS 1 and ITS 2 regions, including the 5.8s gene, of nuclear ribosomal DNA (nrDNA) which occurs between ITS1 and ITS2 (figure 1). Further, the ITS sequences have been demonstrated to be very useful in phylogenetic reconstruction due to relative high numbers of phylogenetically information characters (e.g., Small, et al. 1998). The most recent similarities are assumed to be shared derived characters that are not present in their distant ancestors and are assumed to indicate common ancestry. Thus, the pattern of nucleic acid sequence similarities will be used to imply the genetic relationships within the Verbenaceae, str.

We used the classic methodologies of cladistics to do this. These will include at least maximum parsimony, neighbor-joining, and quartet-puzzling algorithms. The hypothesized relationships (which are shown as branched trees or cladograms) are based on symplesioporphies (primitive characters which are shared by two or more taxa), synapomorphies (a derived characters that are shared by two or more taxa, and held to reflect common ancestry and autopomorphies (a derived character unique to that single taxon). In these methodologies, the algorithms build a progressive bifurcation of a lineage where two taxa are more closely related to each other if they share a more recent common ancestor. A series of hypothesized relationships, they can be represented in a branched diagram called a cladogram. The algorithms usually construct numerous trees but the shortest or simplest are considered to be the most probable (reliable).
The goal is, therefore, to create a cladogram that will show taxa that share common ancestors, and their descendants (monophyletic groups). We wish to avoid polyphyletic assemblages (in which taxa are placed together even though they do not share a common genetic lineage) in a classification scheme.

**Materials and Methods**

We used different protocols of DNA extraction and purification from leaves since no single extraction method is equally effective on all leaf material. Verbenaceae leaves commonly contain quantities of secondary metabolites like polyphenolics and/or polysaccharides which can interfere with the PCR chemistry. These compounds co-precipitate with DNA and are difficult to remove from an extract. Consequently, an extraction protocol was optimized for each species.

The typical extraction protocol was as follows. A specimen of dry or fresh leaf material (approximately 10 mg of dry leaf or 50 mg of fresh leaf tissue) was ground into a fine powder. To the leaf powder was added about 500 µL of a lysis buffer like CTAB solution (e.g. Doyle and Doyle 1987) or a proprietary lysis buffer such as DNAzol™ (Molecular Research Center, Cincinnati OH) with its protocol. Another DNA extraction kit utilized was the MasterPure™ plant DNA kit (Epicenter Inc., Madison WI) in a 1.5 ml microfuge tube. The tube containing the tissue is placed in a heating block at 65°C to accelerate the destruction of the cell organelle membranes by detergents. To the extracts from dry leaf samples we added proteinase K to digest proteins during the membrane
degradation step and RNase to digest RNA. After incubating for at least 30 min. at 65°C, the pigments and remaining proteins were removed using chloroform/isoamyl-alcohol (24:1 v/v). This solution is called CIAA solution. The aqueous solution was mixed well with an equal volume of CIAA and centrifuged for 5-10 min. at 14,000 X g. The photosynthetic pigments go into solution in the CIAA (the lower phase in the tube after centrifugation). The cell debris and proteins formed a layer at the aqueous solution/CIAA interface. The aqueous phase above contained the DNA. The aqueous layer is transferred to a new 1.5 mL microfuge tube. An equal volume of Isopropanol (2-propanol was then added and thoroughly mixed to precipitate the DNA. The tube was then centrifuged for about 2-5 minutes. White to clear pellet contains the DNA plus any other co-precipitated molecules. The pellet was then washed with 70% ethanol to remove water-soluble contaminates without allowing the DNA to go into solution. After a brief centrifugation 70% ethanol solution is decanted. Another brief centrifugation follows to remove remaining 70% ethanol with a pipetter. The pellet was then dried and the pellet re-dissolved in 25-50 µl of water or TE buffer. An aliquot of the extracted DNA was then electrophoresed in a 0.8% HMP agarose gel to determine whether DNA was present and the degree of fragmentation. The DNA was visualized with ethidium bromide. If DNA was present and appeared to be of sufficient quality, a PCR reaction was attempted to amplify the ITS region. Primers 5* (J. Janovec, personal comm.) and 4 of White et al. (1990) were used. Again, an aliquot of the PCR reaction is electrophoresed in a 0.8% HMP agarose to determine whether or not amplification had occurred and to assess the quantity of product. Five to ten µl of PCR product were electrophoresed in a 0.8% agarose gel with ethidium bromide. After approx. 15 min. the
PCR product was visualized with 365nm UV light. A slice of gel containing PCR products was cut out and placed in a 1.5 ml microfuge tube. The Geneclean III kit™ (Bio 101, Inc., San Diego, CA.) protocol was used to recover the purified PCR product. If the PCR product is of sufficient quality and quantity, the product is then directly sequenced.

Direct sequencing of the PCR product used the Sequitherm XL™ II sequencing kit (Epicentre Technologies, Madison, WI). The protocol used fluorescent-labeled sequencing primers (Licor Corp., Lincoln, NE). Sequencing primers used were 5*, and 4 and 6 and 3 primers which are internal primers which are shown in Table 1 (Yuan & Küpfen, 1995).

Cycle sequencing (2 protocols) was used to create the actual sequences. The profile of the first protocol was 10 cycles one minute at 98°C, (2) cooling to 40°C at a rate of 0.5°C/sec., (which is the primer annealing step), and (3) the temperature was increased to 65°C for five min., for extension. The second protocol was (1) 30 cycles of 98°C for 1 min., 48°C for 30 sec., and 72°C for 45 sec. The former protocol is used when “stops” are especially common. An aliquot of 3.0 µL of stop solution is then added to each tube.

The sequencing reactions, after denaturation at 95°C for 3 min., are then loaded onto a 66 cm 3.5% polyacrylimide gel and sequenced using a Licor 4200 sequencer and electrophoresed for approximately 4 h. The sequences are manually read. The sequences are recorded, aligned, and a Nexus file containing the aligned sequences of all taxa created. PAUP 4.0 algorithms will create the cladograms using the algorithms and conditions specified. The algorithms to be used for cladogram generation are still undecided since the methodology is typically determined a posteriori.
Results and Discussion

To date sequencing reactions of 26 taxa have been done. We are currently re-sequencing the following recalcitrant taxa: Citharexylum fructicosum, Bouchea pathulata, Duranta mutsii, Petrea sp., Priva lappulacea, Rehdera trinervus, and Verbena halei,. ITS sequences have been taken from GenBank.

The cladogram generated is shown in figure 2. It suggests that there are two distinct large clades and a small basal clade within Verbenaceae. The first is composed of the genera Aloysia, Lantana, Lippia, Phyla, Priva, and Tamonea while the second clade is composed of Glandularia, Junellia, Stylodon, and Verbena. The genus Verbena, until recently, included Glandularia. Both appear as in the second clade. The third clade is composed of the genera Bouchea and Stachyta. Other studies suggest that Stachytarpheta is within the Verbenaceae clade (Oxelman et al. 1999; Schwartzbach and McDade 2002). It is interesting to note that the data suggest that Lippia graveolens and Phyla nodiflora closely allied to each other and some authorities have used Phyla nodiflora and Lippia nodiflora as synonyms (Herbari Vitual, 2003). A second conclusion is that Stylodon and Junellia are closely related to the genus Verbena. Genera recently removed from Verbenaceae into the Lamiaceae are Callicarpa, Clerodendrum, Tetraclea, and Vitex. The cladogram, based on ITS sequences, indicates that they indeed are related and belong to the Lamiaceae. Numerous other New World Verbenacean taxa have not yet been sampled. Rhedera and Aegiphila have been sampled and the DNA extracted. They have not, however, been sequenced to date. Relatively few genera have not been sampled at least once most notably Nashia (from the Caribbean), and Hierobotana and
Phylogeny of New World Verbenaceae.10

Urbania (from South America). Our conclusions remain highly tentative until unsampled genera are included.

Acknowledgements

I thank the Bridges to the future program for making this possible. Thanks to Nick Lannuti and Dr. Cabeza for their work in the program, Thanks to Dr. Ed Freeman for his mentoring, Sharoon Martinez and Raul Gutieres for their help in the Lab. This study was made possible through the financial support of NIH Grant # 5R25GM49011-5- and NIH Grant # 2R25GM49011-04-
References


Figure legends

Figure 1 - ITS and 5.8s regions

Figure 2 - Verbenaceae Cladogram  Quartet-puzzle
Figure 1
Phylogeny of New World Verbenaceae.
<table>
<thead>
<tr>
<th>Primers Number</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5*</td>
<td>GGA AGG AGA AGT CGT AAC AAG</td>
</tr>
<tr>
<td>3</td>
<td>GCA TCG ATG AAG AAC GCA GC</td>
</tr>
<tr>
<td>4</td>
<td>TCC TCC GCT TAT TGA GC</td>
</tr>
<tr>
<td>6</td>
<td>GGA CGC CCA GGC CCA GGC AGA CGT G</td>
</tr>
</tbody>
</table>

Table 1  ITS Primers
www.tamuk.edu  Lantana camara

Stachytarpheta jamaicensis

www.csdl.tamu.edu

Aloysia gratissima

www.csdl.tamu.edu/FLORA/imaxxvrb.htm

Verbena rigida

www.berrybot.org

http://www.csdl.tamu.edu

Clerodendrum sp.

www.virtualherbarium.org
Phylogeny of New World Verbenaceae.