

GENERIC STATUS OF THE ENDEMIC *Atalantia linearis* Merr (RUTACEAE) BASED ON *rps16* INTRON DATA (cpDNA), WITH A PRELIMINARY REPORT ON ITS PHYTOCHEMICAL COMPONENTS

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ABSTRACT

Rutaceae (or citrus family), specifically subfamily Aurantioideae, includes many economically important species due to their wide array of secondary metabolites. *Atalantia linearis* (Blanco) Merr., an imperfectly known endemic member of the Rutaceae from the Philippines, is a species of shrub found only in the province of Rizal. It was originally described as *Limonia linearis* and later transferred to the genus *Atalantia*. Subsequent study recognized *A. linearis* as a species of *Severinia* based on morphology. Comparative morphology between the two genera shows disparate features. To determine the generic status and position of *A. linearis* with more certitude, *rps16* sequences of two samples were newly generated and analyzed together with previously published related sequences. Surprisingly, parsimony analysis of the aligned 950 base pairs of the *rps16* dataset showed that the two *A. linearis* isolates did not group to neither *Atalantia* nor *Severinia* species included in the analyses. Instead, the two *Atalantia* isolates formed a lineage of its own closely related to the subclade of *Severinia* and another species of *Atalantia* with a moderate support (BS=87). The phytochemical tests showed the presence of sugars, steroids, flavonoids, alkaloids, coumarins, tannins and phenolic compounds in the leaf and stem ethanolic extracts of *A. linearis*. Thus, the endemic species should be explored as a potential source of medicine.

Keywords: *Atalantia linearis*, *rps16* intron, Rutaceae, phytochemical, *Severinia linearis*

INTRODUCTION

Rutaceae, commonly known as the citrus family, comprises about 160 genera and 1,900 species of trees and shrubs (Swingle and Reece, 1967). The family is mostly tropical and subtropical in distribution and is native to Africa, Australia, North and South America and Asia. Rutaceae also contains many members of economic importance. Most notable are those species of *Citrus* L. that produce both the citrus fruits of commerce (lemons, oranges, mandarins, tangerines, limes, kumquats, etc.) and the essential oils used in perfumery (etheral oil and neroli oil of sour orange). The family Rutaceae contains seven subfamilies which includes the Aurantioideae. The Aurantioideae are grouped into two tribes with 33 recognized genera (Swingle and Reece, 1967). Tribe Citreae contains three subtribes with 28 genera, while tribe Clauseneae contains three subtribes with five genera. Both Engler (1931) and Swingle and Reece (1967) believed that Clauseneae contains the more primitive genera of the subfamily based on morphology.

Atalantia linearis belonging to the tribe Citreae is endemic to the Philippines. It is distributed only in the province of Rizal on Luzon island. It was originally named by Blanco (1837) as *Limonia linearis* in the first edition of Flora de Filipinas. However, in Blanco's (1845) second edition of the Flora de Filipinas, he synonymized *Limonia linearis* under *Limonia macrophylla*. It was Merrill (1906) who redescribed it under *Atalantia linearis*, adopting the specific name of Blanco's *Limonia linearis*. The most recent nomenclatural change was that of Swingle (1938) who transferred *Atalantia linearis* to *Severinia linearis*. All these classifications were based on traditional taxonomic methods using morphology. Through the support of molecular data, the study intends to resolve the phylogenetic position of *Atalantia linearis* under family Rutaceae. This study will contribute significantly to the Philippine plant diversity by resolving species complexity by molecular means.

A reconstruction of robust phylogenies that includes representatives of the subfamily Aurantioideae based on molecular data is needed to resolve the true identity of *A. linearis* and to settle its phylogenetic position in Rutaceae family. The chloroplast DNA *rps16* intron region was used in this study because this region indicated somewhat slower sequence evolution than that of the nuclear ribosomal DNA ITS (internal transcribed spacer) region, and provides a valuable source of data for phylogenetic studies (Baker *et al.*, 2000). Various studies have proven that this gene

give good resolution at the generic and species levels (Baker *et al.*, 2000; Oxelman *et al.*, 1997; Wallander and Albert, 2000).

MATERIALS AND METHODS

Taxon Sampling

Field collection of *Atalantia linearis* was conducted at Wawa dam, Rodriguez, Rizal. Morphological characters that are lost upon drying were noted in the field. At least three flowering and/or fruiting branches were collected for herbarium specimens and leaves for silica drying prior to DNA extraction were collected. A total weight of 800 g each of leaves and stems were also collected for the phytochemical screening.

DNA Extraction, PCR Amplification, and Sequencing

Total genomic DNA was extracted from silica-gel dried leaves following the protocol of DNeasy Plant Mini Kit (Qiagen, Germany). The extracted DNA from the two isolates was then amplified via polymerase chain reaction (PCR). The PCR cocktails were mixed as follows (25 μ L): 15.3 μ L nuclease free water, 2.5 μ L 10x PCR buffer, 2.0 μ L MgCl₂, and 1.5 μ L 2 mM dNTP, 1.0 μ L forward and reverse primers, respectively, 0.2 μ L of *Taq* DNA polymerase, and 1.5 μ L DNA. The primer pair used to amplify and sequence is *rpsF* (5' GTG GTA GAA AGC AAC GTG CGA CTT 3') and *rpsR2* (5' TCG GGA TCG AAC ATC AAT TGC AAC 3') (Oxelman *et al.*, 1997). PCR reactions were run on a MJ Research Gradient Thermal Cycler. The program was set to have initial denaturation for 90 s at 97^oC, followed by 35 cycles of 20 s 95^oC for secondary denaturation, 90 s 54^oC for the annealing of primers, 1 min 30 s 72^oC for the primer extension, and finishing with 72^oC for 7 min for the DNA to be incubated and to fill up incompletely extended strands. PCR reactions were repeated until enough volume of each samples were obtained. PCR products were cleaned with Qia-Quick PCR Purification kit (Qiagen, Germany). The amplified and purified DNA bands of the two *Severinia* isolates run in agarose gel electrophoresis. The sequencing procedure was conducted using ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) at the Department of Plant Systematics Bayreuth, Germany. All sequencing was performed on an ABI Prism Model 310, version 3.0 sequencer.

Data Analysis

The *rps16* gene sequences were assembled and edited manually using the Perkin Elmer Sequence Navigator, v. 1.0.1. New sequences and downloaded sequences from EMBL were aligned using the MacClade software version 4.0. The parsimony analyses of the *rps16* data sets (excluding uninformative characters) were performed with Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0b (Swofford, 2002) on a Power Macintosh G3 computer using heuristic searches, with the MULTREES option on, tree-bisection-reconnection (TBR) branch swapping, swap on best only in effect, and 5000 random addition sequences. In all analyses, characters were given equal weight, and gaps were treated as missing data (Simmons and Ochoterena, 2000). The length of the tree (L), consistency index (CI) (Kluge and Farris, 1969) and retention index (RI) (Farris, 1989) were calculated to estimate homoplasy. Bootstrap (BS) (Nakhleh *et al.*, 2005) values were performed to assess relative support for identified clades using 5000 replicates, the MULTREES option off, nearest neighbor interchanges (NNI) branch swapping, and five random addition sequences. Clades receiving a bootstrap support of 86-100% were treated as strongly supported, 70-85% as moderately supported and 50-69% as weakly supported.

Phytochemical Screening

Preparation of the plant material for extraction of organic material was done following the protocol of Aguinaldo *et al.* (2002). Thin Layer Chromatography (TLC) plates used for the experiment were pre-coated silica gel G plates (Merck Art 1.05327^R) cut with dimensions of 2 cm x 5 cm.

Preparation of the Developing Chamber

The developing chambers used were glass jars tightly fitted with covers. The glass jars were lined around the sides with filter paper. This was done to facilitate equilibration with the solvent and to prevent “edge effect.” Enough solvent was poured along the filter paper to moisten it. Formic acid solvent was added to about 10 mm high in the jar. The chamber was equilibrated for 15-30 min.

Sample Application

A capillary tube was used in applying liquid samples into the TLC plates. The capillary tube was dipped into the liquid sample and was filled to a height of about 20 mm. The capillary was positioned at right angles to

the plate and subsequently spotted with the extract about 15 mm from the lower edge of the plate. Spots were air dried between application and the spot diameter was maintained at 2 mm (at most 5 mm) and the distance was maintained not less than 15 mm between neighboring spots.

Development of Chromatogram

The spotted plates were placed in the equilibrated chamber and the points of application were ensured to be above the surface of the solvent. The chamber was then covered tightly. The solvent was allowed to travel up until the solvent reached the mark prepared at the top of the coating. The developed chromatogram was then removed from the chamber and the position of the solvent front was immediately marked. The developed chromatogram was allowed to air-dry and was prepared for visualization.

Visualization of the Chromatogram

A suitable glass sprayer with a fine spray nozzle was loaded with the appropriate reagent to be used for the specific test. The lists of compounds tested were listed in Table 1. Spray reagents were used to the developed TLC plate under hood. The TLC plates were continuously sprayed until desired reaction occurs. No fixed volume of reagent was used. For the specific test for steroids and coumarins, the developed TLC plates were observed under UV light of short wave (240 nm) and long wave (365 nm) after spraying with suitable reagent.

Table 1. List of compounds tested and the specific spray reagent for each class of compounds.

Constituents	Spray Reagent	Standard	Observable Result for a Positive test
Steroids	Acetic anhydride	Cholestrol	Fluorescent colors under UV
Phenols, Tannins Flavonoids	Potassium ferricyanide	Rutin	Blue Spots
Alkaloids	Dragendorff's reagent	Dita (<i>Alstonia scholaris</i>)	Brown-orange visible spots immediately on spraying; colors are not stable.

Sugars	α -Naphthol-sulfuric acid	Rutin	Blue Spots
Coumarins	Methanolic potassium hydroxide (Borntrager reagent)	Turmeric (<i>Curcuma longa</i>)	Coumarins react to form blue (UV 365 nm) colored zones

Preparation of Standards

Rutin was used as standard because it contains a glycoside between the flavonol quercetin and the disaccharide rutinose. Rutin structure contains hydroxyl functional group. *Alstonia scholaris* (Dita) leaves were used as a standard for the detection of indole alkaloids (Macabeo *et al.*, 2005), while *Curcuma longa* (luyang dilaw/Turmeric) was used as a standard for the presence of coumarin (Iranshahi *et al.*, 2009).

RESULTS AND DISCUSSION

New rps16 sequences

Out of the 26 *rps16* sequence data used in the study, two new complete *rps16* sequences for two isolates of *Atalantia linearis* (AL01a and AL01b) were provided and submitted to the European Molecular Biology Laboratory (EMBL) for accession numbers.

Length comparison of rps 16

The length of the assembled 26 *rps16* sequences varied from 801 base pairs (bp) (*Pleiospermium latialatum*) to 849 bp (*Clausena excavata*) with an average length of 824 bp. This is within the range of earlier reported *rps16* sequences (*rps16*:784 - 946bp) (Kelchner, 2002). The total *rps16* base frequencies of the two *Atalantia linearis* isolates range from 801-849 bp.

The position and identity of Atalantia linearis

The aligned *rps16* data matrix included a total of 910 positions. Of the 910 positions, 134 (14.72%) were variable and 74 (8.13%) were parsimony informative characters. The parsimony analysis of *rps16* data

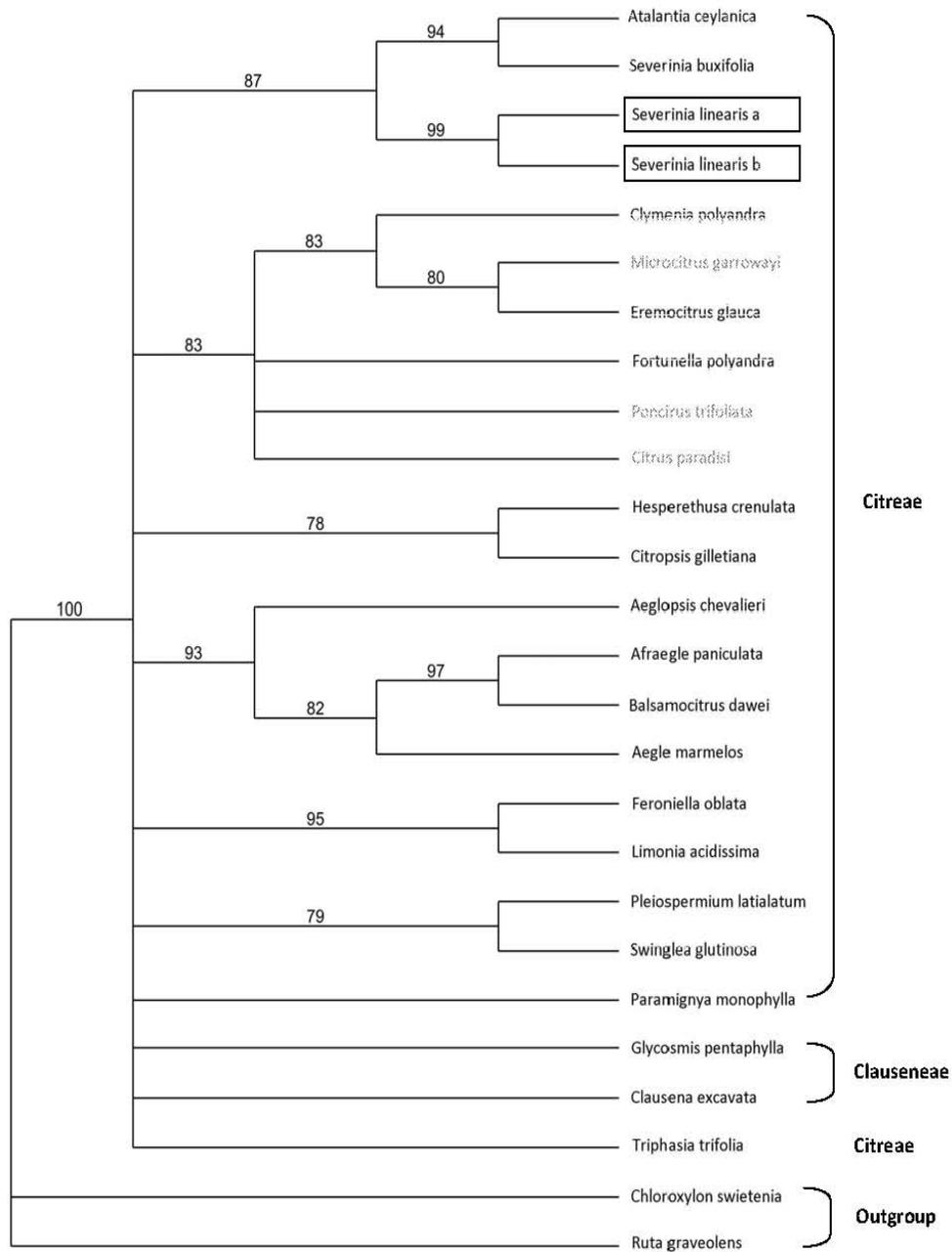


Figure 1. Strict consensus tree derived from 1537 equally parsimonious trees based on the analysis of *rps16* dataset. Numbers above correspond to the bootstrap values >50%

resulted in 1,537 equally parsimonious trees (L= 274, CI = 0.847, and RI = 0.742). The strict consensus tree generated from the *rps16* dataset is shown in Fig. 1. In the *rps16* tree, the two isolates of *A. linearis* are nested within the tribe Citreae (subfamily Aurantioideae) with a strong support (BS=100%). Surprisingly, parsimony analysis of the aligned *rps16* dataset showed that the two isolates of *A. linearis* did not group to neither *Atalantia* nor *Severinia* species included in the analyses. Instead, the two *Atalantia* isolates formed a subclade of its own closely related to the subclade of *Severinia* and *Atalantia* with a moderate support (BS=87%). It could have been better to verify these groupings if more species of *Atalantia* and *Severinia* were included. However, each genus has only one species (*Atalantia ceylanica* and *Severinia buxifolia*) with available *rps16* sequence in the GenBank. Nevertheless, based from the *rps16* tree, there are two taxonomic possibilities that can be inferred. One is *Atalantia linearis* may not be a true member of either *Atalantia* or *Severinia*, the other possibility is the proposal of subsections within the genus *Severinia*. These assumptions could be verified through further morphological examinations and by sequencing other nuclear or chloroplasts markers of *Atalantia* and *Severinia* species for additional support.

Preliminary Phytochemical Screening of A. linearis

Atalantia linearis contained several secondary metabolites based on the phytochemical screening conducted. The successive extracts of stems and leaves of *A. linearis* revealed the presence of alkaloids, flavonoids, sugars, steroids, phenols, coumarins and tannins as shown in Table 2. The presence of different secondary metabolites in plants explains the enormous uses of plants. Some of the functions of these metabolites include their role in the interaction of the plants with its environment, functions in plant's defense system and its role in reproduction as insect attractant. They are also used to determine different aspects of food quality like taste and flavor and most importantly they are also used in the production of dyes, flavors, insecticides and medicines. Secondary metabolites truly are a source of extremely diverse and important number of natural products with industrial and biomedical uses and are interesting targets for drug design (Baikar and Malpathak, 2010). Several members of the family Rutaceae have been studied for their phytomedicinal properties due to the presence of secondary metabolites in their leaves, stems or root extracts. *Lunasia amara* Blanco which was identified to have quinoline alkaloids have antimicrobial and cytotoxicity activity (Macabeo and Aguinaldo, 2008), *Murraya koenigii* (L.) Sprengel possesses analgesic activities (Goyal *et al.*, 2009), while *Citrus* species have gained more

attention not only due to their antioxidant properties but because of their lipid anti-peroxidation effects that are anti-carcinogenic and anti-inflammatory agents. Flavonoids from *Citrus* has been shown to reduce the risk of heart disease (Ghasemi *et al.*, 2009).

Table 2. Phytochemical compounds present in *Atalantia linearis* extracts

Chemical Constituents	Pure Ethanol (stem)	Pure Ethanol (leaves)
Steroids	+	+
Phenols, Tannins, Flavonoids	+	+
Alkaloids	+	+
Sugars	+	+
Coumarins	+	+

Key: + = present, - = absent

The presence of these secondary metabolites in *Atalantia linearis* further suggests that the endemic species is a potential source of a wide array of medicinal applications.

CONCLUSION AND RECOMMENDATIONS

Parsimony analysis of the *rps16* dataset showed that the two isolates of *Atalantia linearis* did not group to neither *Atalantia* nor *Severinia*. Further morphological and molecular studies of *A. linearis* as well as a wider taxon sampling are needed to obtain full support in terms of its generic placement. Consequently, further comprehensive studies of *A. linearis* are much needed to strongly resolve its true identity. The compounds present in the leaf and stem ethanolic extracts of *A. linearis* are sugars, steroids, flavonoids, alkaloids, coumarins, tannins and phenolic compounds. The present study recommends to: (1) sequence more markers (e.g., *atpB-rbcL* spacer, *trnL-trnF* and ITS region) to obtain better taxonomic resolution of *A. linearis*, and (2) conduct a more specific test (e.g., NMR spectroscopy and UV-Vis spectroscopy) to identify the secondary metabolites present in *A. linearis*.

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