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Phylogenetic relationships in *Myrceugenia* (Myrtaceae) based on plastid and nuclear DNA sequences

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ABSTRACT

Myrceugenia is a genus endemic to South America with a disjunct distribution: 12 species occurring mainly in central Chile and approximately 25 in southeastern Brazil. Relationships are reconstructed within *Myrceugenia* from four plastid markers (partial *trnK-matK*, *rpl32-trnL*, *trnQ-5′rps16* and *rpl16*) and two ribosomal nuclear regions (ETS and ITS) using maximum parsimony and Bayesian analyses. Relationships inferred previously from morphological data are not completely consistent with those from molecular data. All molecular analyses support the hypothesis that *Myrceugenia* is monophyletic, except for *M. fernadeziana* that falls outside the genus. Chilean species and Brazilian species form two separate lineages. Chilean species form three early diverging clades, whereas Brazilian species are a strongly supported monophyletic group in a terminal position. Least average evolutionary divergence, low resolution, short branches, and high species diversity found in the Brazilian clade suggest rapid radiation. Geographical distributions and phylogenetic reconstructions suggest that extant *Myrceugenia* species arose in northern Chile followed by colonization southward and finally to the Juan Fernández Islands and southeastern Brazil.

1. Introduction

Myrceugenia O. Berg is a South American genus consisting of about 40 species, which exhibit a disjunct distribution: 26 species occur in southeastern Brazil and adjacent regions of Paraguay, Uruguay and northeastern Argentina, 12 are found in Chile and Andean region of southwestern Argentina and two are endemic to the Juan Fernández archipelago (Landrum, 1981a, 1981b). *Myrceugenia* includes trees and shrubs with 4-merous flowers, usually persistent bracteoles, 2–4 locular ovaries, and few to several ovules per locule (Landrum, 1981a).

Relationships with other members of tribe Myrteae are not clear. Based on embryo structure *Myrceugenia* has been included in the subtribe Myrciinae, but both inflorescence and floral characters are similar to other Myrteae subtribes, Myrtinae and Eugeniinae. In fact, many *Myrceugenia* species were originally included under *Eugenia* L. and *Myrtus* L. (Landrum, 1981a). McVaugh (1968) divided tribe Myrteae into six informal groups according to inflorescence, flower, and seed characters, but he was unable to place *Myrceugenia*, *Luma* A. Gray, *Nothomyrcia* Kausel (a monotypic genus for *Myrceugenia fernandeziana* Hook. et Arn.) and five other genera into any group. He considered these genera to have arisen from the same ancestor as his six larger groups, but to have been less successful and therefore less species rich. Landrum (1981a) "tentatively" accepted three subtribes with in the Myrteae based on embryo structure and offered reason why these "may be true". He listed seven anatomical, morphological and floral characteristics all found in at least one genus in each subtribe and also found in the anomalous genus *Luma*. He speculated that the ancestral group that would link the three subtribes together would have these characteristics and "provisionally" accepted these characteristics as plesiomorphies and the genera that have them as being similar to the ancestor of each subtribe. Thus, he hypothesized that *Myrceugenia* is similar to the ancestor of the subtribe Myrciinae. *Myrcianthes* would hold a similar position in the Eugeniinae and *Blepharocalyx* in the Myrtinae. *Luma* was also hypothesized to belong to this group of ancestral genera. Subsequent molecular work by Lucas et al. (2007) indicates that the Myrteae should be separated into seven groups. One of these is the "Myrceugenia group" including *Myrceugenia*, *Blepharocalyx* and *Luma*. However this group has low support and only appears when nuclear and chloroplast markers are combined.

Species relationships within *Myrceugenia* have been investigated by Landrum (1981b) based on morphological characters. He used various methods and proposed various hypotheses and

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concluded that "there have very probably been three or more cases of transcontinental migration in *Myrceugenia*". Landrum (1981b) provisionally used two species from the Juan Fernández Islands as operational ancestors for his parsimony analysis, but stated that the results should be considered undirected as the species were accepted only "provisionally in order to use the program" and "ignored in the final result." For a final hypothesis based on numerical methods and general knowledge of the ecology of the species and suspected cases of hybridization, Landrum (1981b) chose a point where branches to the two species of Juan Fernández Islands meet, based on their inflorescence structures.

Analyses based on flavonoids (Ruiz et al., 1994) and genetic divergences (Ruiz et al., 2004) have confirmed that the Juan Fernández species evolved in different lineages. These analyses, however, did not support the relationship between *M. fernandeziana* and Brazilian species, but instead this species and *Myrceugenia schultzei* Johow were more related to other Chilean species.

In recent years great advances have been made in molecular systematics (Felsenstein, 2004). This study of the molecular phylogeny of *Myrceugenia* was undertaken with the expectation that these methods, when applied to *Myrceugenia*, would result in a phylogenetic hypothesis with greater support than could be derived from morphological data. The present study is directed toward answering the following questions: What are the phylogenetic relationships among *Myrceugenia* species? Are the species of *Myrceugenia* in Brazil a lineage completely distinct from those of Chile or did they have a common evolutionary history? What is the relationship between the endemic Juan Fernández species and those of the continent?

2. Materials and methods

2.1. Taxon sampling

A total of fifty taxa were analyzed, with two individuals being sampled in *Blepharocalyx salicifolius* (Kunth) O. Berg and *Myrceugenia ovata* (Hook. and Arn.) var. *regnelliana* (O. Berg) Landrum. All species from Chile (12 species, including two varieties) and many from Brazil (25 species including nine varieties) were investigated. Eight species were included as outgroups: *Luma apiculata* (DC.) Burret, *Luma chequen* (A. Gray), *Blepharocalyx cruckshanksii* (Hook. and Arn.) Nied., *B. salicifolius*, all of these belonging to the "Group Myrceugenia" (Lucas et al., 2007), *Myrcianthes cisplatensis* (Cambess.) O. Berg, *Ugni molinae* Turcz, *Ugni selkirkii* (Hook. and Arn.) O. Berg, and *Myrtus communis* L., the last one having been recognized as the sister group of tribe Myrteae (Lucas et al., 2007). All DNA sequences analyzed were obtained during this study. Table 1 gives voucher specimens, herbarium, country of origin, and GenBank accession numbers.

2.2. DNA extraction

Total DNA genomic was extracted mainly from herbarium specimens, in some cases from silica-gel dried or fresh leaves, using a modified CTAB (Cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987). Ground material was previously treated several times with sorbitol, followed by incubation at 65–70 °C for 30 min with CTAB buffer and Sarkosyl. The precipitated material was left overnight at −20 °C and centrifuged at 14,000 rpm for 30 min, then followed by two washes with 70% ethanol. Total DNA was resuspended in 30–50 μL of 1% TE buffer. Some samples were extracted using DNeasy extraction kit (Qiagen) according to the manufacturer's instructions.

2.3. Marker selection and primer design

Nine chloroplast markers were evaluated (*matK* gen and flanking *trnK* intron, *ndhA* intron, *rpl16* intron, intergenic spacer *rpl32-trnL* and flanking *rpl32* gen, and the intergenic spacer *psbA-trnH*, *psbD-trnT*, *TrnL-trnF*, *trnQ-5′rps16*, following Shaw et al., 2007), but all of these showed low variability. However, we selected the four most variable regions (partial *trnK-matK*, *rpl32-trnL*, *trnQ-5′rps16* and *rpl16*) for reconstructing phylogenetic relationships among species of *Myrceugenia*. Internal transcribed spacer (ITS: ITS1-5.8S-ITS2) of nuclear ribosomal genes 18S and 26S and the external transcribed spacer 18S-26S rDNA (ETS) were also included. Primers used for each region are listed in Table 2. Two internal primers for region *trnQ-5′rps16* were designed.

2.4. Amplification, sequencing and alignment

ITS sequences were amplified mainly in a volume of 25 μL containing 2.5 units of *Taq* polymerase (Paq5000 DNA polymerase, Stratagene Inc.), 2.5 μL 10X PCR buffer (Stratagene Inc.), 0.2 mL of each dNTP (25 mM), 0.5 μL of each primer (4 μM). All other sequences were amplified using 18 μL 1.1X ReddyMix PCR Master Mix (Thermo Fisher Scientific Inc, ABGene, UK), 0.4 μL of each primer (20 μM), 0.6 μL 0.4% of bovine serum albumin (BSA, MBI-Fermentas, St. Leon-Rot, Germany), and 1–2 μL template DNA. For reducing secondary structure problems 0.5 μL of dimethyl sulfoxide (DMSO) were added to all nuclear markers amplifications. PCR conditions for ITS were according to the Stratagene polymerase manufacturer's recommendations. An initial DNA denaturation at 95 °C for 2 min was followed by 30 cycles 95 °C for 20 s, 48–52 °C for 20 s, and 72 °C for 20 s, then a final extension at 72 °C for 5 min. PCR conditions for ETS were according to Lucas et al. (2007). An initial denaturation at 94 °C for 5 min was followed by 30 cycles 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, then a final extension at 72 °C for 5 min. PCR conditions for chloroplast regions were according to Shaw et al. (2007) with some modifications. A first template denaturation at 80 °C for 5 min was followed by 35 cycles 94 °C for 30 s, 50–53 °C for 30 s, and a ramp of 0.3 °C/s to 65 °C, then 65 °C for 3 min, finally an extension at 65 °C for 8 min.

All amplicons were run on a 1% agarose gel to confirm amplification of PCR products. ITS amplified products were purified with QIAquick PCR purification kits (Qiagen) according to manufacturer's recommendations. All other PCR products were purified using 0.5 μL Exonuclease I and 1 μL Thermosensitive alkaline phosphatase, FastAP (Fermentas) by incubation at 37 °C for 45 min, and later at 80 °C for 15 min. Most amplified ITS products were sent for sequencing either to Macrogen (Korea) or to the Molecular Lab at the University of Santiago de Chile. All others sequences were sequenced in the Molecular Laboratory of the Department of Systematic and Evolutionary Botany in the Biodiversity Center of the University of Vienna. For cycle sequencing a 10 μL reaction volume including 1 μL of the primer (3.2 μM), 0.5 μL of BigDye Terminator v3.1 Ready Reaction mix (Applied Biosystems, Austria), 1.75 μL of sequencing buffer, 4 μL of amplified purified product, and 2.75 μL of ddH_2O was used. Cycle sequencing parameters consisted of an initial 96 °C for 1 min, followed by 35 cycles 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, finally 60 °C for 5 min. Electrophoresis was performed on a 3730 DNA analyzer (Applied Biosystems, ABI). All sequences were analyzed and edited with Lasergene Seqman Pro 7.1.1 (DNASTAR) to obtain consensus sequences from both forward and reverse DNA strands. It was not possible to sequence either *Myrceugenia bracteosa* (DC.) D. Legrand and Kausel, *Myrceugenia hoehnei* (Burret) D. Legrand and Kausel, *Myrceugenia pilotantha* (Kiaersk.) Landrum var. *nothorufa* (D. Legrand) Landrum, *Myrceugenia scutellata* D. Legrand, *Myrceugenia venosa* D. Legrand