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ESTUDO FILOGENÉTICO DAS ESPÉCIES DA SEÇÃO *TORVA* DO GÊNERO *SOLANUM* L. (SOLANACEAE) NA REGIÃO SUL DO BRASIL

Dissertação de Mestrado

Rogéria Beatriz Miz

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Estudo Filogenético das Espécies da Seção *Torva* do Gênero *Solanum* L. (Solanaceae) na Região Sul do Brasil

Rogéria Beatriz Miz

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Orientadora: Dra. Tatiana Teixeira de Souza Chies

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Instituições

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Sumário

Resumo

O gênero *Solanum* L. (Solanaceae) compreende mais de 1000 espécies, incluindo táxons de grande interesse econômico por seu valor alimentício e medicinal. Este gênero é dividido em três subgêneros: *Bassovia*, *Solanum* e *Leptostemonum*. O subgênero *Leptostemonum é* dividido em dez seções, e entre essas destaca-se a seção *Torva* que possui representantes no sul do Brasil, e cujas espécies têm amplo interesse por apresentarem substâncias ativas de grande utilidade farmacológica. Entretanto, dentro dessa seção existem problemas taxonômicos, inclusive com a presença de indivíduos de morfologia intermediária, que dificultam sua classificação e, conseqüentemente, o seu melhor aproveitamento. Nesse trabalho, foram realizados dois estudos de caráter filogenético a fim de conhecer as relações de parentesco entre as espécies de *Solanum* seção *Torva,* presentes no sul do Brasil, e destas com espécies de outras seções do subgênero *Leptostemonum*. Em ambos os estudos foram utilizados quatro marcadores (genomas nuclear e plastidial): a região ITS (espaçadores internos transcritos do DNA nuclear ribossomal) incluindo ITS1, ITS2 e o gene 5,8S; o íntron *trn*L e os espaçadores intergênicos *trn*L-*trn*F e *trn*S-*trn*G do DNA plastidial. O marcador ISSR (Inter Simple Sequence Repeats) foi utilizado para verificar a variabilidade genética entre as espécies de *Solanum* seção *Torva* e testar o grau de polimorfismo de quatro "primers" dentro dessa seção. As análises realizadas evidenciaram uma origem monofilética para a seção *Torva*. Além disso, foi verificada uma relação de parentesco mais acentuado dessa seção com *S. melongena*, *S. jamaicense* e *S. sisymbriifolium.* Dentro da seção *Torva* foram observados agrupamentos que relacionam a espécie de morfologia intermediária a seus possíveis progenitores *S. paniculatum* e *S. guaraniticum*. Os quatro agrupamentos mais freqüentes observados dentro da seção foram: a aproximação de *S. guaraniticum*, *S. bonariense* e *S. paniculatum* X *S. guaraniticum*; o relacionamento entre *S. adspersum* e *S. tabacifolium*; a interação entre *S. paniculatum* e a espécie de morfologia

intermediária; e a aproximação entre *S. paniculatum* e *S. variabile*. Este trabalho contribuiu para o conhecimento evolutivo das espécies dessa complexa seção que vem levantando interesse de inúmeros pesquisadores.

Palavras chaves: Solanaceae; gênero *Solanum;* seção *Torva;* filogenia; marcadores nucleares e plastidiais; marcadores ISSR.

Abstract

The genus *Solanum* L. (Solanaceae) comprises more than 1,000 species, including taxa with large economic importance for its nutritive and medicinal values. This genus is divided into three subgenus: *Bassovia*, *Solanum* and *Leptostemonum*. The subgenus *Leptostemonum* of *Solanum* is divided in ten sections and, among them, the section *Torva,* that possess species form the south of Brazil, which showed large interest by its active substance of large pharmacological utility. However, this section presents some taxonomic problems, including individuals with intermediate morphology, that difficult its identification and its utilization. In this work, it were realized two studies of phylogenetic character, in the attempt to understand the relationship among species of the section *Torva* of *Solanum,* and among the section *Torva* with species of other sections of the subgenus *Leptostemonum*. In these studies it were used four markers (nuclear and chloroplast genome): the nuclear ribosomal internal transcribed spacer region (ITS) including ITS1, ITS2 and 5,8S gene; the *trn*L intron, and *trn*L-*trn*F, *trn*S-*trn*G intergenic spacers of the cpDNA. The ISSR (Inter Simple Sequence Repeats) marker was used to examine the genetic variability among the species of the section *Torva* of *Solanum*, and to test the polymorphism in the section. The analysis showed the section *Torva* as monophyletic, and it was found a close relationship of this section with *S. melongena*, *S. jamaicense* and *S. sisymbriifolium.* The section *Torva* showed assembly the species with intermediate morphology with its supposed parents *S. paniculatum* and *S. guaraniticum*. The four relationships more frequently showed within the section were: the assembly among *S. guaraniticum*, *S. bonariense* and *S. paniculatum* X *S. guaraniticum*; the relationship among *S. adspersum* and *S. tabacifolium*; the relationship among *S. paniculatum* and the intermediate species; and the relationship among *S. paniculatum* and *S. variabile*. This work was helpful to understand the evolution of the species belonging to this section that have being of high interest of many researchers.

Key words: Solanaceae; genus *Solanum;* section *Torva*; phylogeny, nuclear and plastidial markers; ISSR marker.

1.- Introdução

Considerações Gerais

1.1.- Família Solanaceae e o gênero *Solanum* L.

[U1] **Comentário:** Citar nome do autor

A família Solanaceae possui uma grande importância econômica, agrícola e farmacêutica. Compreende aproximadamente 93 gêneros e 2300 espécies (Hunziker, 2001). Segundo Hunziker (2001), a família Solanaceae subdivide-se em seis subfamílias: Cestroideae, Juanulloideae, Solanoideae, Salpiglossoideae, Schizanthoideae e Anthocercidoideae. Dentre as seis subfamílias, somente as três primeiras têm representantes nativos na região sul do Brasil. A distribuição da família mostra-se cosmopolita, com o principal centro de diversidade taxonômica e endemismo na América do Sul. Várias espécies desta família movimentam grandes somas de recursos financeiros em todo o mundo, sendo algumas alimentícias, tais como: *Solanum tuberosum* L. (batata-inglesa), *Solanum melongena* L. (berinjela), *Lycopersicum esculentum* L. (tomate), *Capsicum anuum* L. (pimentão), além de outras de interesse regional e ornamental como as petúnias. Além disso, compreende um grande número de espécies com propriedades tóxicas e farmacológicas, desempenhando, desta forma, uma grande importância na medicina e na produção de drogas terapêuticas (Roddick, 1991). A indústria farmacêutica utiliza produtos do metabolismo secundário de várias espécies de *Solanum* como fonte de substâncias ativas: alcalóides tropânicos, vitanolídeos e alcalóides esteroidais. Os alcalóides esteroidais encontrados em *Solanum* são utilizados na produção de esteróides farmacêuticos e também se mostram comprometidos com compostos antitumorais e com agentes que auxiliam contra a esquistossomíase (Roddick, 1991).

O gênero *Solanum* compreende mais de 1000 espécies em todo o mundo (D'Arcy, 1991). Este gênero pertence à subfamília Solanoideae, tribo Solaneae, subtribo Solaninae. *Solanum* é quinto maior gêneros dentre as Angiospermas (*Magnoliophyta*), distribuído em todas as regiões tropicais e subtropicais das Américas, África e Austrália, com um menor número de espécies euroasiáticas. Dunal (1852) dividiu o gênero *Solanum* em duas seções, *Pachystemonum* e *Leptostemonum*, os quais foram elevados a subgênero *Solanum* e *Leptostemonum*, respectivamente, por Bitter (1912, 1913, 1919). Segundo Nee (1999), o gênero está dividido em três subgêneros: *Bassovia*, *Solanum* e *Leptostemonum.* Segundo Mentz (comunicação pessoal), na região sul do Brasil o gênero *Solanum* apresenta 93 espécies nativas distribuídas nos três subgêneros: *Bassovia,* com 13 espécies, *Solanum,* com 49 espécies, e *Leptostemonum,* com 31 espécies.

O subgênero *Lepostemonum*, que será tratado neste estudo, é dividido em dez seções (Nee, 1999): *Polytrichum*, *Melongena*, *Erythrotrichum*, *Crinitum*, *Herposolanum*, *Micracantha*, *Torva*, *Acanthophora*, *Persicariae* e *Lasiocarpa*. As espécies pertencentes a este subgênero caracterizam-se principalmente por serem espinhentas e possuírem tricomas simples e quase sempre estrelados.

1.2.- Seção *Torva*

Uma das seções do subgênero *Leptostemonum* é a seção *Torva,* criada por Nees (1834), com base em *S. torvum* Sw. (Figura 1- F), espécie originária do México, América Central e norte da América do Sul. A seção *Torva* é composta por 40 espécies (Nee, 1999), as quais possuem flores na maioria das vezes perfeitas e relativamente numerosas em grandes inflorescências, sendo pequenos a grandes arbustos. Estas espécies estão centralizadas nas Américas, com alguns representantes na África, Nova Guiné e Pacífico. Para o Brasil são citadas oito espécies sendo quatro pertencentes à Região Sul (Nee, 1999). Além das espécies, citadas por Nee, Witasek (1910) também menciona *S. adspersum,* que ocorre no Brasil, na região litorânea dos estados de São Paulo e Paraná. Portanto, são citadas

ao todo para região sul do Brasil cinco espécies pertencentes à seção *Torva*: *Solanum paniculatum* L., *Solanum guaraniticum* A. St.-Hil*., Solanum adspersum* Witasek, *Solanum variabile* Mart. e *Solanum tabacifolium* Dunal (Mentz, 2004).

1.2.1- *Solanum paniculatum* (Figura1-A)

Popularmente é conhecida como jurubeba, jupeba, juribeba, jurupeba, gerobeba e joá-manso. A Farmacopéia Brasileira reconhece esta espécie como a verdadeira "jurubeba" (Farmacopéia Brasileira, 1929). Ocorre no Paraguai e, no Brasil, principalmente, acompanhando a costa Atlântica do Rio Grande do Norte ao Rio Grande do Sul, em todas as formações vegetais, às vezes também como ruderal e cultivada. Na Argentina, foi citada pela primeira vez na flora por Schinini e López (2000).

Esta espécie apresenta plantas perenes reproduzidas por sementes, com arbustos de até 2,5 metros de altura (ramificado). Caracterizam-se morfologicamente por possuírem folhas solitárias, apresentarem tricomas estrelados, sésseis nos ramos apicais, pela presença de acúleos engrossados e alargados na base, pela inflorescência cimosa com muitas flores e pelo fruto glabro, globoso, de coloração amarela. Além disso, esta espécie apresenta longos rizomas subterrâneos, dos quais emergem caules adventícios, formando clones que podem ser bem amplos. Portanto, as plantas que ocorrem em um determinado local tendem a ser semelhantes, apesar do grande polimorfismo que existe nesta espécie (Mentz e Oliveira, 2004).

São plantas utilizadas como medicinais e em rituais afro-brasileiros. Na farmacopéia popular são utilizadas as folhas, frutos e raízes, no preparo de infusões. Atribui-se à planta efeitos febrífugas, e estimulante das funções digestivas e do fígado. No Brasil, *Solanum paniculatum* é comumente utilizada na medicina folclórica para o tratamento de doenças do fígado e gastrointestinais (Pio Corrêa, 1984). Estudos recentes com camundongos apresentam uma associação direta da atividade antiúlcera dos extratos metabólicos de *Solanum paniculatum* com a atividade anti-secretora do suco gástrico, validando o uso folclórico da planta para o tratamento de

doenças gástricas (Mesia-Vela e cols., 2002). Do ponto de vista químico, são encontradas saponinas e alcalóides, cujos compostos apresentam algum efeito tóxico.

Segundo Mentz e Oliveira (2004), é possível que as populações de *Solanum paniculatum* presentes no Rio Grande do Sul tenham tido introdução recente, pois quase todas as exsicatas diferem das populações coletadas da Ilha de Santa Catarina para o norte. Além disso, foi verificada a existência de possíveis híbridos entre *Solanum paniculatum* e *Solanum guaraniticum* (Figura 1-C), essa constatação se deve, a maioria das populações, observadas em Porto Alegre e municípios vizinhos, terem flores brancas, com anteras praticamente desprovidas de grãos de pólen e raramente produzindo frutos (Mentz e Oliveira, 2004).

1.2.2- *Solanum guaraniticum* (Figura 1-B)

Solanum guaraniticum é conhecida popularmente como jurubeba ou falsa-jurubeba. Saint Hilaire nomeou esta espécie provavelmente relacionando a região dos povos indígenas do sul do Brasil. Esta espécie tem ocorrência no Paraguai, Uruguai, Argentina e no Brasil, nos estados de Minas Gerais, São Paulo, Rio de Janeiro, Paraná, Santa Catarina e Rio Grande do Sul. Na região sul ocorre em todas as formações vegetais, apresentando também comportamento ruderal.

Esta espécie apresenta-se como arbusto ereto, de até 2 metros de altura, com floração e frutificação durante todo o ano. Caracteriza-se morfologicamente por apresentar ramos basais glabros e apicais cobertos de tricomas pedicelados, estrelados, por possuir acúleos aciculares, raramente alargados na base, folhas solitárias, inflorescência cimosa (5-20 flores) e pelos frutos glabros, globosos, amarelos a amarelo-alaranjados quando maduros.

Solanum guaraniticum, sob o nome de *Solanum fastigiatum*, tem sido citada como responsável por afetar o sistema nervoso central de bovinos, evidenciada no campo por crises tipo epileptiforme, com perda de equilíbrio, quedas e tremores musculares (Riet-Correa e cols., 1983; Paulovich e cols., 2002). Existem alguns

problemas taxonômicos referentes a esta espécie: o nome *Solanum fastigiatum* Willd., citado para o sul do Brasil, corresponde a *Solanum bonariense* L. (Figura 1-E), espécie da Argentina e Uruguai, enquanto que a variedade descrita para *Solanum fastigiatum*, corresponde a *Solanum guaraniticum*. O tipo de *Solanum fastigiatum* Willd. var. *acicularium* não difere do tipo de *Solanum guaraniticum* A. St. Hil., sendo válido este último. Já *Solanum guaraniticum* difere morfologicamente de *Solanum bonariense* apresentando este último, ramos glabros, angulosos, vinosos, com acúleos curvos, agudos, glabros, brilhantes, folhas com face adaxial quase glabra, com tricomas estrelados sésseis e face abaxial com tricomas estrelados esparsos e inflorescência cimoso-paniculada, sem acúleos (Mentz e Oliveira, 2004).

1.2.3- *Solanum adspersum*

A espécie *Solanum adspersum*, recebeu este nome provavelmente devido ao indumento disperso - do latim “adspersus”, espalhado (Mentz e Oliveira, 2004).

Esta espécie tem ocorrência na região sudeste do Brasil, no estado de São Paulo, e na região sul, no estado do Paraná, em áreas de formações pioneiras litorâneas, clareiras e orla da mata, bem como em capoeiras.

Esta espécie é um arbusto ereto de até 2,5 metros de altura e possui um período de floração entre julho e dezembro e de frutificação entre agosto e dezembro. Caracteriza-se morfologicamente por possuir ramos lenhosos basais glabros e os apicais cobertos de tricomas sésseis, pela presença de inflorescência cimosa com 10-25 flores, sendo as 5-8 basais férteis e frutos globosos de cor amarela quando maduros (Mentz e Oliveira, 2004).

1.2.4- *Solanum variabile* (Figura 1-D)

A espécie *Solanum variabile* é conhecida como falsa “jurubeba” no Brasil. Segundo Smith e Downs (1966), também pode ser chamada de jurubeba-velame.

Além deste, também é chamada popularmente como velame, velame-de-capoeira, juveva e jupicanga.

No Brasil esta espécie tem ocorrência nos estados de Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul (Comissão Geográfica e Geologia, 1972; Leitão Filho, 1972; Pio Côrrea, 1984; Nee, 1999). *Solanum variabile* também é encontrada em alguns países vizinhos como Paraguai e Uruguai (Smith e Downs, 1966; Sacco, 1985).

Solanum variabile é um arbusto ereto ou arvoreta de até 4 metros de altura, possue um período de floração entre agosto e maio e de frutificação entre setembro e junho. Esta espécie é bastante diversificada morfologicamente, apresenta desde folhas inteiras e mais estreitas, até folhas largas e lobadas, sendo que em algumas plantas ocorrem muitos acúleos, enquanto em outras esses são muito raros. Caracteriza-se também pelos seus ramos densamente cobertos de tricomas estrelado-pedicelados, com folhas solitárias, pela inflorescência cimosa e pelos seus frutos glabros, globosos, alaranjados quando maduros (Mentz e Oliveira, 2004).

Segundo trabalho recente (Antonio e cols., 2004), o extrato etanólico obtido das partes aéreas de *Solanum variabile* tem um significante efeito preventivo e curativo, em úlceras duodenais.

1.2.5- *Solanum tabacifolium*

Solanum tabacifolium foi mencionada por Smith e Downs (1966), sob o nome de *Solanum asperolanatum* Ruiz e Pavón. Esta última ocorre nos países da costa ocidental da América do Sul, como Colômbia, Equador, Peru e Venezuela (Mentz e Oliveira, 2004). O nome *Solanum tabacifolium*, devido às regras de nomenclatura, é um nome ilegítimo e precisa ser substituído. Segundo Mentz e Oliveira (2004), Michael Nee (The New York Botanical Garden) está propondo um novo nome, ainda inédito para esta espécie. Popularmente, a espécie é conhecida como juveva, jurubeba ou cardo-branco (Smith e Downs, 1966). No Brasil, é encontrada na Região Sudeste (Minas Gerais e São Paulo), e na Região Sul (Paraná e Santa Catarina), na

região das Florestas Ombrófilas Densa e Mista, na região da Floresta Estacional Semidecidual, em borda de matas e em terrenos alterados.

Essa espécie é uma arvoreta de até 4 metros de altura, possue um período de floração e frutificação de junho a dezembro. Caracteriza-se morfologicamente por possuir ramos cobertos de tricomas estrelados, com acúleos pequenos, levemente alargados na base, folhas solitárias ou geminadas (desiguais no tamanho), inflorescência cimosa com flores unilaterais em cada ramo e frutos glabros, globosos, amarelos quando maduros (Mentz e Oliveira, 2004).

São plantas citadas como útil no tratamento de doenças do fígado (Smith e Downs, 1966).

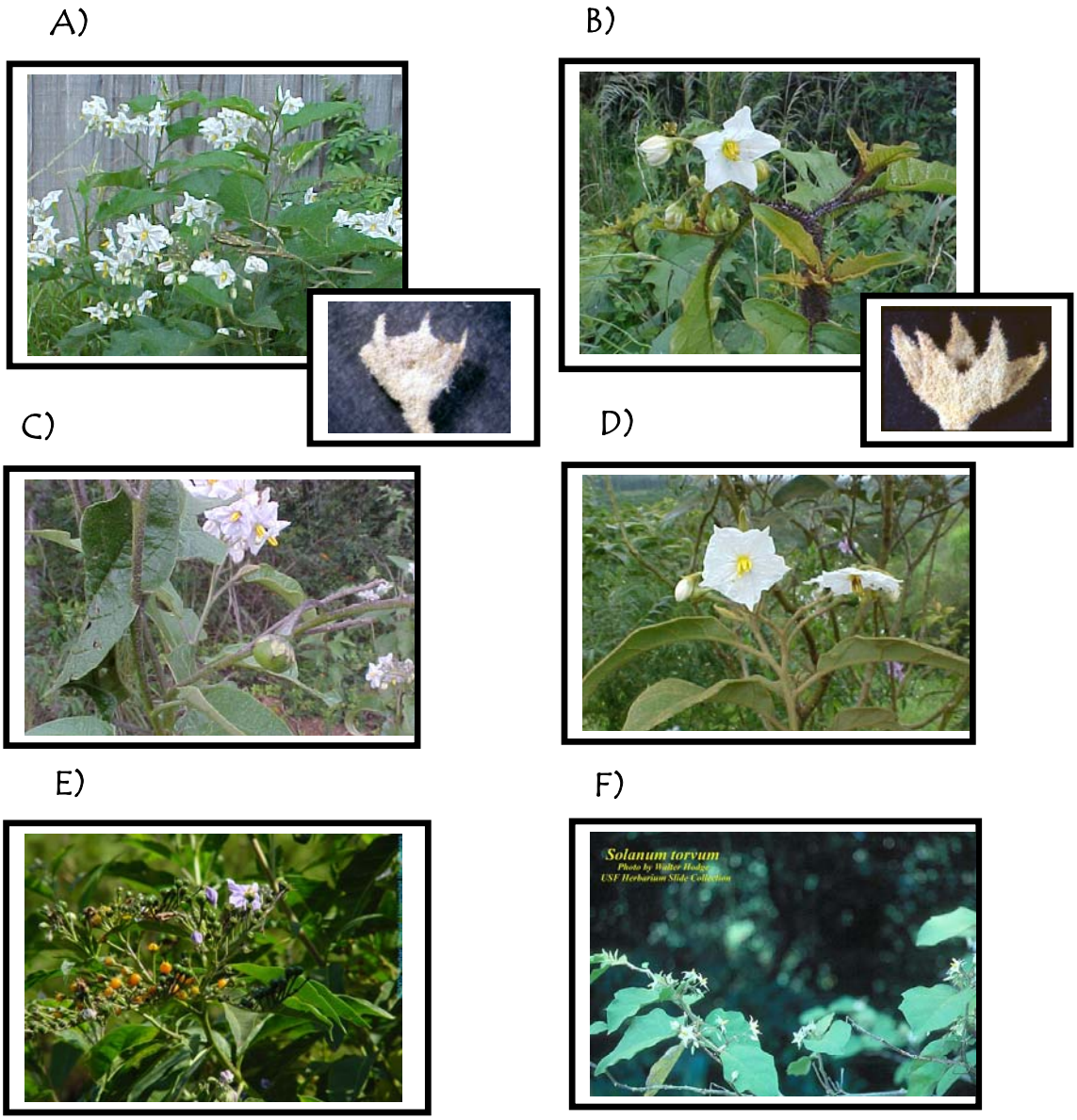

Figura 1: Foto de algumas espécies de *Solanum* seção *Torva,* incluídas neste trabalho. A) *S. paniculatum* (detalhe do cálice); B) *S. guaraniticum* (detalhe do cálice); C) *S. paniculatum* X *S. guaraniticum*; D) *S. variabile*; E) *S. bonariense*; F) *S. torvum*. As fotos de A – D foram cedidas por Lílian Mentz.

1.3.- Marcadores Moleculares em Filogenia de Plantas

As Filogenias moleculares são baseadas nas ferramentas da Biologia Molecular e da Cladística para acessar o conhecimento das relações evolutivas entre as espécies. Esta área do conhecimento tem crescido amplamente desde o início dos anos 1990, devido ao desenvolvimento dos mais rigorosos métodos de construção de árvores, combinado com a explosão das informações de seqüências de DNA. A importância e utilização da filogenia molecular têm aumentado, tanto pelo sucesso na aplicação de reconstruções de árvores, como pelas novas técnicas filogenéticas que surgem a cada dia com o advento da Bioinformática, para resolver um dos mais complexos problemas em biologia.

A classificação taxonômica das espécies vegetais baseava-se, até pouco tempo, principalmente em características morfológicas; mais recentemente, caracteres moleculares passaram a constituir uma fonte rica de informação taxonômica.

Com o desenvolvimento de técnicas e reagentes para seqüênciamento direto, o uso da informação contida nas seqüências de DNA de certos fragmentos tornou-se viável para estudos envolvendo maior número de táxons. Diferentes fragmentos de DNA têm sido utilizados como fonte de caracteres para a análise filogenética, sendo os espaçadores e/ou íntrons os fragmentos ideais para o estudo infragenérico, pois acumulam mutações mais rapidamente do que regiões codificantes, graças a pressões de seleção mais baixas atuando sobre eles (Matioli, 2001). Assim, essas regiões ficam mais livres para variar, fornecendo informações filogenéticas suficientes para evidenciar eventos evolutivos relativamente recentes (Small e cols., 1998).

Um dos fragmentos mais utilizados para inferir filogenia são os espaçadores internos transcritos – ITS (internal transcribed spacers) do DNA ribossomal nuclear (nrDNA), que correspondem aos espaçadores ITS1 e ITS2, os quais separam os genes 18S, 5,8S e 26S (Figura 2). Segundo uma pesquisa realizada por Alvarez e Wendel (2003), no período entre 1998 e 2003, mais de 65% de todos os estudos

filogenéticos de plantas incluíram seqüências de ITS. A popularidade deste marcador se deve principalmente pela facilidade em gerar dados, pois existem primers universais para a amplificação da região ITS (Baldwin, 1992). Esta vantagem leva à geração de muitos dados em pouco tempo. Além disso, existem milhares de cópias do nrDNA por genoma, facilitando a amplificação das mesmas. A região ITS tem provado ser suficientemente variável para resolver relações filogenéticas em diferentes grupos de plantas em níveis taxonômicos mais baixos (Baldwin e cols., 1995), como os infragenéricos, devido a sua alta taxa de substituição nucleotídica (Baldwin, 1992; Baldwin e cols., 1995).

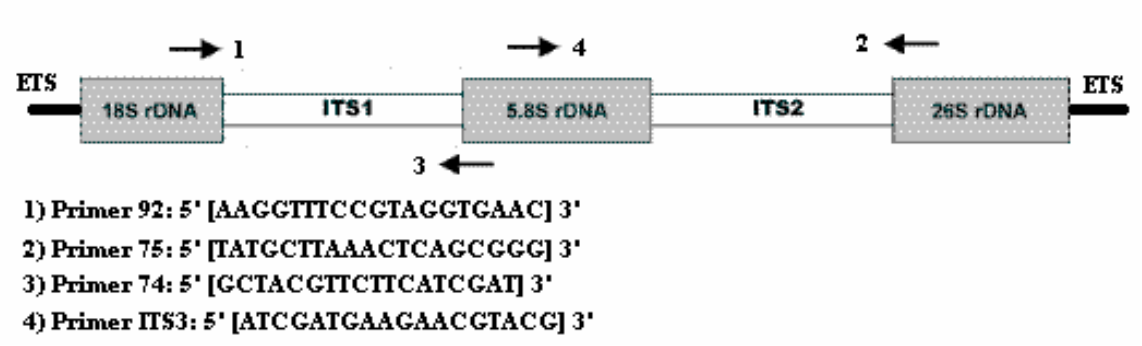

Figura 2: Representação esquemática das posições e direções dos “primers” universais usados para amplificar e seqüenciar as regiões intergênicas ITS 1 e ITS 2 do nrDNA.

Ultimamente, a maioria dos trabalhos envolvendo filogenias moleculares tem utilizado mais de um fragmento para inferir as relações de parentesco entre os táxons e, a escolha tem sido feita principalmente com fragmentos contidos nas regiões do DNA de cloroplasto ou plastidial (cpDNA). Um dos motivos da escolha do cpDNA é, também, a facilidade em se encontrar regiões caracterizadas e com primers universais descritos. Um outro motivo que tem levado a combinação de ambos os genomas (nuclear e plastidial) em análises filogenéticas é o tipo de

herança envolvido na origem destes genomas. O DNA plastidial geralmente apresenta herança uniparental materna, em Angiospermas, enquanto o DNA nuclear possui herança biparental. Portanto, a utilização de marcadores moleculares de ambas origens auxilia na complementaridade dos dados a serem analisados e pode ajudar na elucidação da origem de híbridos naturais. No trabalho, envolvendo análise filogenética, realizado por Miz (2003) pode-se observar que apesar dos espaçadores ITS terem sido mais informativos que os fragmentos de cpDNA, esses marcadores mostraram-se muito mais informativos quando foram analisados em conjunto. Segundo Baldwin e cols. (1995), é necessária a utilização de dados do genoma nuclear para suplementar e enriquecer os dados obtidos com o seqüenciamento de fragmentos do cpDNA. O autor comenta que tais necessidades são devidas, entre outros fatores, a informações muitas vezes insuficientes nas árvores baseadas em cpDNA, já que o genoma plastidial é um dos mais conservados entre os três genomas vegetais. Baldwin, entretando, deixa claro que o seu objetivo não é depreciar as filogenias com base no cpDNA, e, sim, apenas demonstrar o insuficiente potencial de se estimar a filogenia de um organismo baseado em uma única organela genômica ou gene nuclear.

Entre os fragmentos de cloroplasto descritos destaca-se a região do íntron do gene *trn*L e o espaçador intergênico *trn*L-*trn*F (Taberlet e cols., 1991), os quais vem sendo co-amplificados (Shaw e cols., 2005) e o espaçador intergênico *trn*S-*trn*G (Hamilton, 1999) ilustrados na figura 3. Esses fragmentos têm sido utilizados em análises combinadas de vários trabalhos para melhorar a resolução filogenética. Em estudos recentes, eles foram utilizados para inferir a filogenia de diferentes seções do subgênero *Leptostemonum* do gênero *Solanum* (Bohs, 2004; Levin e cols., 2005), mostrando altos índices de consistência e de suporte para a topologia das árvores. No estudo realizado por Shaw e cols. (2005), constata-se que entre 21 regiões não codificantes espalhadas pelo cpDNA, os espaçadores *trn*L-*trn*F e *trn*S-*trn*G estão entre as cinco regiões que provêm maior número de caracteres potencialmente informativos.

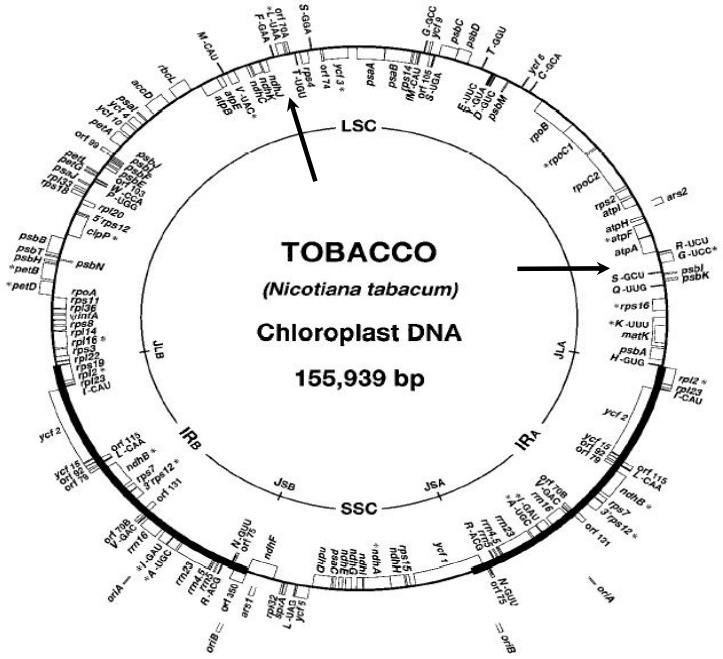

Figura 3: Genoma do cpDNA de *Nicotidiana tabacum*. As setas mostram as regiões do íntron *trn*L e dos espaçadores intergênicos *trn*L-*trn*F *e trn*S-*trn*G.

1.4- Utilização de Marcadores Moleculares do tipo ISSR para caracterização genética de espécies vegetais

Uma das técnicas que vem sendo amplamente utilizada para estudos de variabilidade genética é o ISSR (Inter Simple Sequence Repeats) que foi proposta pela primeira vez por Zietkiewicz e cols. (1994). O ISSR é um método baseado na amplificação de DNA por PCR, que envolve a amplificação de fragmentos de DNA

presentes em uma distância amplificável entre dois SSRs ou microssatélites idênticos repetidos (Figura 4), orientados em direções opostas (Reddy e cols., 2002). Assim sendo, nessa técnica os SSRs (Seqüências Simples Repetidas), também chamados de microssatélites, são utilizados como “primers” para amplificar principalmente regiões entre SSRs (Reddy e cols., 2002). A técnica do ISSR-PCR permite, portanto, a detecção de polimorfismos em *locus* localizados entre os microssatélites, utilizando seqüências simples repetidas (SSRs) como “primers” (Zietkiewicz e cols., 1994; Wu e cols., 1994). A mudança da taxa evolutiva dentro dos microssatélites é considerada maior que em outras regiões do DNA. Conseqüentemente, a probabilidade de polimorfismo dessas seqüências é maior. No caso do ISSR, a fonte de variabilidade pode ser atribuída à amostra do DNA, à natureza do “primer” utilizado ou ao método de detecção (Reddy e cols., 2002).

Os produtos resultantes da amplificação do tipo ISSR segregam como marcadores mendelianos dominantes simples (Gupta e cols., 1994; Tsumura e cols., 1996; Ratnaparkhe e cols., 1998; Wang e cols., 1998). Entretanto, eles também têm se mostrado como marcadores codominantes em alguns casos, permitindo assim distinção entre homozigotos e heterozigotos (Wu e cols., 1994; Akagi e cols., 1996; Wang e cols., 1998; Sankar e Moore, 2001). O polimorfismo é evidenciado quando há presença e ausência de um fragmento (bandas) após a amplificação e eletroforese em gel.

Os ISSR têm sido utilizados em análises genéticas, para verificar a variabilidade intra e intertaxonômicas, associados a outros marcadores como RAPD e RFLP (Davierwala e cols., 2000; Panda e cols., 2003). Esses marcadores vêm sendo utilizados igualmente para reconstruções filogenéticas em níveis de espécies ou acima (Xu e Sun, 2001; Yockteng e cols., 2003). Além disso, são igualmente aplicados em estudos de “fingerprinting” (Charters e Wilkinson, 2000), mapeamento genômico (Becker e Heun, 1995), seleção assistida por marcadores (Ratnaparkhe e cols., 1998; Hussain e cols., 2000), determinação da freqüência de motivos SSR (Nagaoka e Ogihara, 1997; Blair e cols., 1999; Varghese e cols.,

2000) e estudos de populações naturais e especiação (Wolff e Morgan-Richards, 1998).

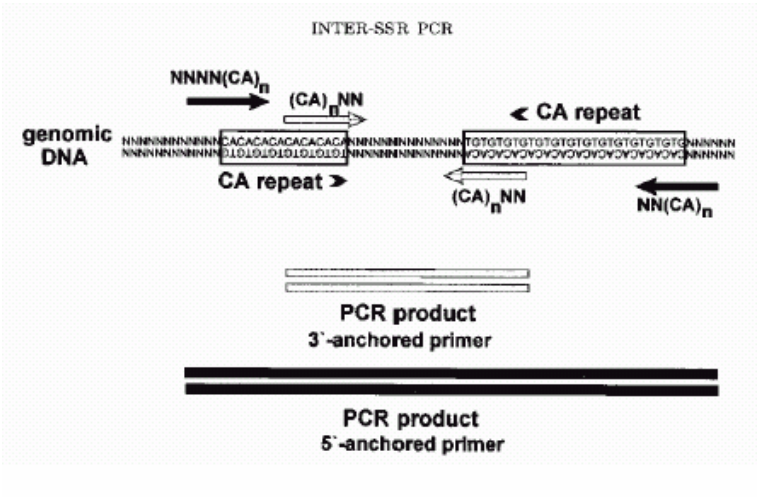

Figura 4: Representação esquemática da obtenção do produto de PCR utilizando a técnica de ISSR (esquema retirado do artigo de Zietkiewicz e cols, 1994).

1.5- Análise dos Dados

1.5.1 Reconstrução Filogenética

Atualmente, estão sendo utilizados quatro métodos principais para inferir as relações evolutivas entre as espécies na busca de árvores filogenéticas: máxima parcimônia (MP), método de distância (MD), máxima verossimilhança (MV) e Inferência Bayesiana (IB), sendo os dois últimos métodos probabilísticos.

A máxima parcimônia consiste na busca da solução mais parcimoniosa, ou seja, a(s) árvore(s) com menor número de mudanças mutacionais, sendo vista como

a hipótese mais econômica. Este tipo de análise tem grande vantagem em relação aos demais métodos de inferência filogenética por ser muito simples, pois escolhe o caminho evolutivo com menos pressupostos. Um dos algoritmos heurísticos mais comumente utilizado para a MP, para casos em que se analisa um grande número de táxons, é o algoritmo *branch swapping* - TBR (tree bisection and reconnection). Nesta opção a árvore é dividida em duas subárvores disjuntas, sendo unidas por um par de ramos diferentes dos originais. Este procedimento é repetido até que todos os pares possíveis de ramos dessas duas subárvores sejam unidos e a melhor árvore seja definida.

Com o método de distância pressupõe-se que todos os caracteres evoluem na mesma taxa e, os dados são estimados como distâncias pareadas entre as seqüências nucleotídicas. O algoritmo mais utilizado para este método é o Neighbor-Joining, NJ (Saitou e Nei, 1987), o qual leva em consideração as taxas evolutivas distintas e faz parte do grupo de métodos de evolução mínima, no qual a árvore com a menor soma total de ramos é procurada.

Os métodos probabilísticos MV e IB são estimados segundo um modelo evolutivo pré-determinado. Ambos os métodos tem o objetivo de encontrar uma topologia de árvore e correlacionar parâmetros associados, que aumentem a probabilidade de conter um conjunto de dados de acordo com o modelo proposto. A máxima verossimilhança tem sido amplamente utilizada para inferir filogenia de espécies vegetais. Nesse método a probabilidade deve ser calculada para todas as topologias possíveis, e a árvore combinando topologia mais comprimento de ramos que apresentar a maior verossimilhança (probabilidade) é considerada a melhor estimativa da filogenia. A Inferência Bayesiana baseia-se em uma metodologia estatística antiga e parecida com a MV, mas foi pouco utilizada até recentemente. A sua principal vantagem em comparação à MV é o tempo de análise, uma vez que permite estimar os parâmetros do modelo pela mesma técnica.

Para testar a confiabilidade das topologias tanto em árvores de distância, como de parcimônia e até mesmo de verossimilhança, o teste mais comumente utilizado é o de *Bootstrap* (Felsenstein, 1985). A base do método consiste de uma simples

reamostragem com reposição pseudoaleatória dos dados. A reamostragem é repetida muitas vezes (geralmente 1000 replicações) e, no final, uma árvore consenso é gerada a partir das melhores árvores obtidas. Um outro teste que vem sendo utilizado para avaliar a confiabilidade das relações filogenéticas é o suporte de Bremer ou índice de decay (Bremer, 1988), o qual determina o número de passos extras em que cada agrupamento se mantém a partir de uma árvore inicial pré-estabelecida.

1.5.2 Métodos de medida de variabilidade

A variabilidade intra e inter taxonômica dos táxons baseados em marcadores do tipo ISSR vem sendo medida verificando-se a similaridade entre os dados qualitativos, utilizando principalmente o coeficiente de Jaccard e de Dice. Estas análises geralmente são realizadas com o pacote NTSYS-pc (Rohlf and Marcus, 1993).

2.- Objetivo Geral

O presente trabalho tem como objetivo principal investigar sobre as relações filogenéticas das espécies da seção *Torva* do gênero *Solanum* presentes na região sul do Brasil, a partir de diferentes marcadores moleculares.

2.1 Objetivos Específicos (Artigo 1)

- Evidenciar as relações filogenéticas entre os táxons da seção *Torva,* e a relação desta com as seções *Acanthophora* e *Lasiocarpa,* e demais espécies pertencentes ao subgênero *Leptostemonum,* e verificar a monofilia destes grupos;
- Avaliar a utilidade de quatro marcadores moleculares do tipo sequências de DNA para o gênero *Solanum*: ITS (rDNA nuclear); íntron do gene *trn*L e os espaçadores *trn*L-*trn*F, *trn*S-*trn*G do cpDNA;
- Verificar quanto à ocorrência de híbridos naturais entre *Solanum paniculatum* e *Solanum guaraniicum*;
- Contribuir para o conhecimento taxonômico do gênero *Solanum.*

2.2 Objetivos específicos (Artigo 2)

- Evidenciar as relações filogenéticas e medir a variabilidade inter taxonômica entre os cinco representantes da seção *Torva* no sul do Brasil, utilizando caracteres do tipo seqüências de DNA (regiões de DNA do cloroplasto e ITS) e o marcador ISSR (Inter Simple Sequence Repeat).

3.- Artigo 1

Phylogenetic Relationships among the Section *Torva* and Related Sections from "Spiny Solanums" (*Solanum* Subgenus *Leptostemonum*, Solanaceae)

(A ser submetido para publicação em "Molecular Phylogenetics and Evolution")

Phylogenetic Relationships among the Section *Torva* and Related Sections from "Spiny Solanums" (*Solanum* Subgenus *Leptostemonum*, Solanaceae)

Rogéria B. Miz [a], Tatiana T. Souza - Chies [a, b]

[a] Universidade Federal do Rio Grande do Sul, Programa de Pós Graduação em Genética e Biologia Molecular, Av. Bento Gonçalves, 9500. Caixa Postal 15053, CEP: 91501-970, Porto Alegre, RS, Brazil. Tel.:+55 51 3316-9830, Fax: + 55 51 3316-7311

[b] Universidade Federal do Rio Grande do Sul, Departamento de Botânica, Av. Bento Gonçalves, 9500. Caixa Postal 15053, CEP: 91501-970, Porto Alegre, RS, Brazil. Tel.:+55 51 3316-7569, Fax: + 55 51 3316-7686

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Excluído: 7311

Corresponding author: **Dra. Tatiana T. Souza-Chies**

Address for correspondence.

Departamento de Botânica, UFRGS

Prédio 43433

CEP: 91501-970, Porto Alegre, RS, Brazil

Tel.: 55 51 3316-7569

Fax: 55 51 3316-7686

E-mail: tatiana.chies@ufrgs.br

Abstract

Solanum section *Torva* is of considerable interest to phytochemists and biological researchers because its representatives contain various active chemical substances with important pharmacological properties although some species provoke toxic effects on cattle. However, the species belonging to the section *Torva* and called as “jurubeba” present some taxonomic problems that make difficult their effective pharmacological use. Because of this, we proposed a study to investigate and elucidate some evolutionary questions related to this section and its phylogenetic relationships with other sections of subgenus *Leptostemonum*. In this study, 80 samples were investigated based on sequence variability of the nuclear ribosomal DNA internal transcribed spacers (ITS), as well as the chloroplast intron *trn*L, *trn*L-*trn*F and *trn*S-*trn*G spacers. Five different matrixes were analyzed as basis for phylogenetic approach. The combined data from the chloroplast analyses formed well supported trees and provided a high index of consistency. *Solanum* section *Torva* was proposed as monophyletic and it is closed to *S. melongena*, *S. jamaicense* and *S. sisymbriifolium*. Furthermore, we agree with other authors who proposed that the subgenus *Leptostemonum* is monophyletic and that *S. wendlandii* must be excluded of the subgenus.

Key Words: Solanaceae; *Solanum* section *Torva*; subgenus *Leptostemonum*; ITS; intron *trn*L plus *trn*L-*trn*F; *trn*S-*trn*G; phylogenetic analysis.

Introduction

Solanaceae corresponds to a large family of Angiosperms that contains many taxa having economical, agricultural, and pharmaceutical importance - the family includes about 92 genera and 2,300 species (Hunziker, 2001). The family is widely distributed with the main centre of taxonomic diversity and endemism located in South America. The genus *Solanum* L. comprises more than 1,000 species (D'Arcy, 1991) and it is distributed worldwide. According to Dunal (1852) *Solanum* is divided into two sections: *Pachystemonum* and *Leptostemonum*, which were respectively, elevated into the subgenus *Solanum* and *Leptostemonum* by Bitter (1912, 1913, and 1919). However, Nee (1999) considered that the genus *Solanum* is divided into three subgenera: *Bassovia*, *Solanum* and *Leptostemonum.* In southern Brazil, the genus *Solanum* comprises about 93 native species, distributed in all three subgenera recognized by Nee (1999): *Bassovia* with 13 species, *Solanum* with 56 species and *Leptostemonum* with 31 species (Mentz, personal communication). The members of the subgenus *Leptostemonum* are also referred as "spiny solanums" because the majority of species have epidermal prickles on stems and/or leaves and most members of this group have stellate hairs and/or simple trichomes (Whalen, 1984; Mentz et al., 2000). The species from the subgenus *Leptostemonum* are distributed within ten sections (Nee, 1999) including *Torva* Nees, *Acanthophora* Dunal and *Lasiocarpa* (Dunal) D'Arcy, which are considered in this study.

Solanum section *Torva* was described based on *Solanum torvum* Sw., a species from Mexico, Central America and North of South America. Morphological characters that define this section include small to large shrubs, mostly perfect and relatively numerous flowers in large inflorescences, young branches and leaves covered by stellate trichomes, and with stem

and leaves armed with spiny at least in early stages. *Solanum* section *Torva* comprises 40 species (Nee, 1999) in the world (centered in the Americas, with scattered representatives in Africa, Asia, New Guinea and Pacific) - eight species are found in Brazil and five of them are present in Rio Grande do Sul State. Some of the species showed taxonomic problems of species delimitation with a morphologically intermediate taxon among two species that complicate its identification. The species from section *Torva* analysed in the present study are *Solanum paniculatum* L., *Solanum guaraniticum* A. St.-Hil. and *Solanum variabile* Mart..

The species of section *Torva* are considered close to species of section *Acanthophora* because their similar morphological characters. Therefore, in this work we included species from *Acanthophora* and *Lasiocarpa* sections of *Solanum*, which, according Nee (1999), are well supported as sister taxa. The main differences are the predominance of simple hairs on the stems and upper leaf surfaces and the glabrous mature fruits of the section *Acanthophora* in contrast to the stellate hairs and pubescent fruits found in the section *Lasiocarpa*. Levin et al. (2005, 2006) and Bohs (2004) agree with Nee (1999) that the *Acanthophora* and *Lasiocarpa* sections have strong support as sister taxa. Notwithstanding the above cited papers including the section *Torva*, the species belonging to this section are little studied despite their taxonomic problems.

Thus, the main goal of the present study were investigate about the phylogenetic relationships of the taxa within the *Solanum* section *Torva* and among it and the *Lasiocarpa* plus *Acanthophora* sections and other closely related members of the subgenus *Leptostemonum*.

To accomplish this goal, phylogenetic relationships were inferred from DNA sequence data from four gene regions. These regions include one from the nuclear genome - the nuclear

ribosomal internal transcribed spacer region (ITS) plus the 5,8S gene - and three of chloroplast data from the *trn*L intron and *trn*L-*trn*F (Taberlet et al., 1991) plus *trn*S-*trn*G (Hamilton, 1999) spacer regions. In addition, we discuss about the monophyly of the three sections, and evaluated the degree to which four different regions were helpful in resolving relationships among closely related *Solanum* species.

Material and Methods

Taxon sampling

The overall taxon sampling included in the present study encompasses 80 samples. The distinct data sets were treated with a different number of taxa (Table 1).

ITS matrix - the ITS matrix was analyzed having 60 samples, the other 20 samples were not analyzed by different reasons including that the sequence for other fragments was get from genbank and there is not available sequence to that DNA region, or there were sufficient number of sequenced samples to represent the taxon. The excluded sequences were: Section *Torva* - one sample of intermediate morphology species, four of *S. guaraniticum,* two of *S. paniculatum* and one of *S. variabile*; section *Acanthophora* - three of *S. atropurpureum*, two of *S. aculeatissimum* and one of *S. vaillantii;* section *Lasiocarpa* - *S. felinum* and *S. pectinatum;* and outgroup - *S. mauritianum* and *S. dulcamara*.

***trn*L-*trn*F plus intron *trn*L matrix** – this matrix was analyzed having 76 samples, the other four samples were not analyzed by the same reasons explained above. The excluded sequences were: Section *Torva* - one sample of *S. variabile*; section *Acanthophora* - *S. agrarium* and one sample of *S. vaillantii*; and outgroup - *S. concinum*.

***trn*S-*trn*G matrix** – the matrix was analyzed having 58 samples, the other 22 samples were not analyzed by the same reasons above. The excluded sequences were: Section *Torva* - one sample of intermediate morphology species, three of *S. guaraniticum*, one of *S. paniculatum* and one of *S. variabile*; Section *Acanthophora* - two samples of *S. atropurpureum*, two of *S. aculeatissimum;* Section *Lasiocarpa* - only four species were included: *S. candidum*, *S. pseudolulo*, *S. quitoense* and *S. stramonifolium.* Other sections of the subgenus *Leptostemonum*: one sample of *S. sisymbriifolium*; and outgroup (no-spiny) - *S. mauritianum*, *S. dulcamara* and *S. concinnum*.

Chloroplast data set combined - the plastid matrix was analyzed with 56 samples, the other 24 samples were not analyzed by the same reasons above. The excluded sequences were: Section *Torva* - one sample of intermediate morphology species, three of *S. guaraniticum*, one of *S. paniculatum* and one of *S. variabile;* Section *Acanthophora* - two samples of *S. atropurpureum*, two of *S. aculeatissimum*, one of *S. vaillantii* and *S. agrarium*; Section *Lasiocarpa* – the same taxon sampling as above. Other sections of the subgenus *Leptostemonum* - one sample of *S. sisymbriifolium*; and outgroup (no-spiny) - *S. mauritianum*, *S. dulcamara* and *S. concinnum*.

All data sets combined – the combined matrix of the three DNA sequences was anayzed with 45 samples. The excluded sequences were: Section *Torva* - two samples of intermediate morphology species, six of *S. guaraniticum*, three of *S. paniculatum*, two of *S. variabile*; Section *Acanthophora* - four samples of *S. atropurpureum*, four of *S. aculeatissimum*, one of *S. vaillantii* and *S. agrarium*; Section *Lasiocarpa* - the same 8 samples excluded of the above matrix. Other sections of the subgenus *Leptostemonum* - one sample of *S. sisymbriifolium*; and outgroup (no-spiny) - *S. mauritianum*, *S. dulcamara* and *S. concinnum*.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from fresh or silica-gel-dried leaves using the CTAB method (Doyle and Doyle, 1990).

ITS – Amplification of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, composed of ITS 1, the 5.8S gene, and ITS 2 (Baldwin, 1992; Baldwin et al., 1995), was done using primers 92 (5'- AAG GTT TCC GTA GGT GAA C-3') and 75 (5' –TAT GCT TAA ACT CAG CGG G- 3') described by Desfeux and Lejeune (1996). Polymerase chain reaction (PCR) conditions were: 1µl of template DNA (30-100 ng), 2.5 µl of reaction buffer 10x, 0.75 µl of $MgCl_2$ (50mM), 0.5 µl of dNTP (10mM), 0.25 µl of *Taq* DNA polymerase (5U/µl), 0.5µl of each primer 92 (204 pmol/µl) and 75 (202 pmol/µl), 2.5 µl of DMSO (96%) and H_2O to a total volume of 25 µl. In the cycle sequencing reactions, DMSO was included because GC-rich regions, and the reactions were realized in thermal cycler Applied Biosystems equipment (Gene Amp PCR System 2400). The DNA amplifications were performed in a thermal cycler using the "Hot Start" PCR method: 94°C for 5min, 72°C for 6 min; 35 cycles at 94°C for 45 sec, 58°C for 1 min, 72°C for 1 min and 30 sec; with a final extension at 72°C for 10 min. The amplified material was purified with enzymatic pre-treatment (Shrimp alkaline phosphatase and exonuclease I, Amersham Biosciences). The PCR products were direct sequenced in an ABI PRISM 3100 automated sequencer with the primers 92 and ITS3 (5'-ATC GAT GAA GAA CGT ACG- 3') also described by Desfeux and Lejeune (1996).

***trn*S-*trn*G** - The chloroplast intergenic spacer between *trn*S and *trn*G was amplified using primers *trn* S (5'- GCC GCT TTA GTC CAC TCA GC- 3') and *trn* G (5'- GAA CGA ATC ACA CTT TTA CCA C-3') of Hamilton (1999). Reactions were obtained with 2.5 μl reaction buffer 10x, 0.5 μl of dNTPs (10mM), 0.8 μl of $MgCl_2$ (50mM), 0.5 μl of each primer, 0.2 μl of *Taq* DNA polymerase (5U/μl), 1 μl of DNA (30 – 50 ng) and H_2O to complete 25 μl. The thermal cycler program included an initial denaturing at 94°C for 5 min; 40 cycles at 94°C for 1 min, 49°C for 1 min, 72°C for 1 min; ending with an extension at 72°C for 10 min. PCR products were cleaned and sequenced as before, using the same primers as for amplification.

***trn*L-*trn*F plus *trn*L intron –** Each region was treated separately in the phylogenetic analyses. However, the non-coding cpDNA regions *trn*L-*trn*F spacer plus *trn*L intron, are adjacent and were co-amplified as a single contiguous unit. From a cost/benefit perspective, it is best to amplify and sequence both of these regions together instead of separately by maximizing the number of characters obtained per two sequencing reactions (Shaw et al., 2005). Primers used for amplification were ´´c`` and ´´f`` of Taberlet et al. (1991). Reactions of 25 μl were obtained with 2.5 μl reaction buffer 10x, with 0.5 μl of dNTPs (10mM), 0.75 μl of $MgCl_2$ (50mM), 0.5 μl of each primer, 0.25 μl of *Taq* DNA polymerase (5U/μl), 1 μl of DNA, 1 μl of DMSO (96%) and H_2O to complete a volume of 25 μl. The thermal cycler program included an initial denaturing at 94°C for 3 min; 35 cycle at 94°C for 1 min, 55°C for 1min, 72°C for 2 min; ending with an extension at 72°C for 3 min. These two regions were cleaned and sequenced as before using with the same primers that were used for PCR.

Sequence alignment

Sequences were analyzed and edited by Chromas 1.45 version software (McCarthy 1996-1998). The sequence alignments were made using the Clustal X 1.81 program (Thompson et al., 1997) and manually refined with the help of GeneDoc (Nicholas and Nicholas, 1997) and BioEdit softwares (Hall, 1999).

Phylogenetic analyses

The separate (ITS, *trn*S-*trn*G, and *trn*L-*trn*F plus intron *trn*L) and combined (overall plastid data sets or ITS plus plastid regions) phylogenetic analyses were executed using three main methods: (1) maximum parsimony (MP) using heuristic searches with tree-bisection-reconnection (TBR) branch swapping, gaps were treated as fifth base, and the Maxtrees option was used to limit the number of trees, the parsimony analyses were conducted in PAUP 4.0b10 version (Swofford, 2002); (2) Bayesian analysis was performed using MrBayes 3.0b4 by Windows (Huelsenbeck and Ronquist, 2001), to generate a posterior probability distribution using Markov chain Monte Carlo (MCMC) methods, with the evaluation of at least 1,000,000 generations and a 'burn-in' region of 4,000 trees. This analysis follows the model proposed by Akaike information criterion (AIC) test (Akaike, 1974), and was executed using the ModelTest program (Posada and Crandall, 1998); (3) for the distance analysis we used the Neighbor joining (NJ) (Saitou and Nei, 1987) method, according to the model proposed by the ModelTest, in PAUP.

The relative proportion of homoplasy data in the MP analyses was estimated though Consistency and Retention Indexes (CI and RI respectively) available as PAUP tools. The g1 statistics (Hillis, 1991) was calculated from 1,000 random trees generated by PAUP, to

measure the phylogenetic information content of the data sets. The general frequencies of the four nucleotides A, C, G, and T were calculated in PAUP. The resampling Bootstrap test (BS) (Felsenstein, 1985) was performed with 1,000 full heuristic replicates with tree bisection-reconnection (TBR) for MP and neighbor joining analyses in PAUP. For the Bayesian analysis the posterior probability of each clade on the 50% majority rule consensus tree was calculated (Hall, 2001). Data sets congruence was assessed using partition homogeneity (PHT) tests (essentially the incongruence length difference (ILD) test of Farris et al. (1994, 1995), as implemented in PAUP. The partition Bremer support (PBS) scores method (Baker et al., 1998; Bremer, 1988, 1994) was also used to measure the amount of support provided by *trn*L-*trn*F plus intron *trn*L, *trn*S-*trn*G and ITS regions to each node on the total evidence phylogeny. The PBS values were calculated using PAUP and TreeRot.v2 Sorenson (1999).

Results

ITS regions (nuclear data sets) – The ITS sequences matrix presented an aligned length of 727 characters, including ITS1, 5.8 S rRNA gene, and ITS2. Of these 727 characters, 228 were parsimony informative (PI) across 60 samples (Table 2). The general frequencies of the four nucleotides A, C, G, and T were, respectively, 0.17, 0.36, 0.32, and 0.15, showing high guanine-cytosine (GC) content (68%). The g1 statistic for ITS trees was of - 0.449, indicating that for this region the data are significantly skewed and therefore has a substantial phylogenetic signal (Hillis, 1991). In the maximum parsimony analysis, the heuristic search for ITS data was limited by Maxtrees in 20,000 most parsimonious trees with 1,079 evolutionary steps with a consistency index (CI) of 0.55 and retention index (RI) of 0.80

(Table 2). The 50% majority rule consensus tree presents two large groups (Figure 1). The first group (supported by BS 93) is composed by species of *Solanum* section *Torva* and taxa belonging to other sections of the subgenus *Leptostemonum.* In this group the section *Torva* (highly supported by BS 100) is divided into two subgroups formed by individuals of *S. guaraniticum* and individuals recognized as intermediate morphology species (BS 69), the second subgroup is formed by *S. paniculatum* and *S. variabile* species (BS 82). *Solanum torvum* is the most basal species of the section *Torva*. The second group is divided also into two subgroups, (i) formed by species of the section *Acanthophora* (BS 83) and (ii) by the species of the section *Lasiocarpa (*BS 100). *Torva* and *Lasiocarpa* sections proved to be monophyletic, while the section *Acanthophora* is not (due to *S. agrarium* and *S. stenandrum* species) in the ITS analyses. Moreover, this tree showed that the species of the subgenus *Leptostemonum* are all closely related (BS 97) except for *S. wendlandii*, which is found outside the ingroup. The group formed by *S. agrarium* and *S. stenandrum* (species of *Acanthophora*), *S. stagnale* and *S. robustum* is not supported, but in the work of Levin et al. (2006) these species make part of the Robustum clade (informal name) and are highly supported. However, Levin et al. (2006) showed only the trees obtained from total evidence and probably the plastid DNA contributes to support this clade. The selected model for the Bayesian analyses using ModelTest was GTR+I+G with gamma shape parameters estimated of 0.5865. The Bayesian tree topology is very similar to the MP trees using the ITS regions. The distance analysis showed *Solanum* section *Torva* close only to *S. melongena* and *S. jamaicense*, and also confirmed the monophyly of the section *Torva* (BS 98), data not showed.

Chloroplast data

***trn*S-*trn*G –** The *trn*S-*trn*G sequences across 58 taxa showed 87 characters being PI with aligned length of 868 (Table 2). The general frequencies of the four nucleotides A, C, G, and T were, respectively, 0.33, 0.17, 0.14, and 0.36 (GC = 31%). The g1 statistic for *trn*S-*trn*G trees was of - 0.162. In the maximum parsimony analysis, the heuristic search was limited to 20,000 most parsimonious trees with 319 steps, with CI of 0.72 and RI of 0.86 (Table 2). The 50% majority rule consensus tree presents four groups (data not shown). The first group shows species of the section *Torva* closed to the species: *S. sysimbriifolium*, *S. jamaicense* and *S. melongena*. *Solanum* section *Torva* is divided into two subgroups, one showing *S. variabile* close to *S. paniculatum* and the intermediate species (BS 70), and the other showing *S. guaraniticum* related to the intermediate species (BS 98), being this latter group very well supported. The second group is divided into two subgroups where the first one is composed by the section *Acanthophora*, and the other by the section *Lasiocarpa* (BS 98). The third group strongly supported (BS 100) is composed by *S. agrarium* and *S. stenandrum* both of them from the section *Acanthophora*. The fourth group is formed by *S. mammosum* (*Solanum* section *Acanthophora*), *S. robustum* and *S. stagnale*. The group formed by the subgenus *Leptostemonum* presents high support (BS 91) and showed *S. wendlandii* in a basal position. *Torva* and *Lasiocarpa* sections proved to be monophyletic while the section *Acanthophora* is not monophyletic, due to *S. agrarium*, *S. stenandrum* and *S. mammosum* (outside the group formed by this section). The evaluative model selected for the Bayesian analyses was K81uf+G, with gamma shape parameters estimated of 0.3262. The trees obtained from the Bayesian and distance analyses (data not showed) are divided into two large groups: one

including *S. melongena*, *S. jamaicense*, and *S. sisymbriifolium* adjacent to the section *Torva* (94% of support in Bayesian analysis), and the other is formed by one subgroup composed by the section *Acanthophora* species (except for *S. agrarium* and *S. stenandrum* which formed another subgroup). In these analyses *S. mammosum* is included in the group formed by the species of the section *Acanthophora* while S. *robustum* and *S. stagnale* form another subgroup with the section *Lasiocarpa*.

***trn*L-*trn*F plus intron *trn*L**- The *trnL-trnF* intergenic spacer combined with the *trn*L gene intron presented a total alignment length of 1,270 bp, being 216 (PI) across 76 taxa (Table 2). The general frequencies of the four nucleotides A, C, G, and T were, respectively, 0.36, 0.19, 0.16, 0.29 (GC = 35%). The g1 statistic for the trees was of -0.218. In the maximum parsimony analyses the heuristic search was limited to 20,000 (Maxtrees) most parsimonious trees with 640 evolutionary steps with CI of 0.74 and RI of 0.89 (Table 2). The 50% majority rule consensus tree presents two large groups. A group is formed by species of *Solanum* section *Torva* (BS 85) and *S. melongena*. The section *Torva* was divided into two subgroups with *S. torvum* being the most basal species. The first subgroup showed individuals of *S. guaraniticum* and *S. paniculatum* related to the intermediate species; and the second subgroup showed *S. variabile* (BS 91) and *S. guaraniticum* adjacent to the intermediate species (BS 99). The second group formed in the consensus tree is divided into two subgroups. One of them is formed by the section *Lasiocarpa* (BS 53) and the other by the section *Acanthophora* (BS 100) with *S. stenandrum* (a species of the section *Acanthophora*) being the most basal species of these two subgroups. Moreover, it is possible to visualize in the majority rule consensus tree that *S. wendlandii* is outside of the ingroup formed by species of the subgenus

Leptostemonum (BS 97). The evolutive model selected to perform the Bayesian analyses was TVM+I+G, with gamma shape parameters estimated of 0.8590. The Bayesian tree topology proved to be similar to the trees originated by the parsimony analyzes except for the position of *S. jamaicense* that is a sister group of the section *Torva* with *S. melongena*. The distance analyses showed less resolution in the tree than MP and Bayesian analysis for the *trn*L-*trn*F plus intron *trn*L combined region, but support the major groups visualized in other analyses, with support of 78% for the section *Torva*, 96 % for section *Lasiocarpa*, 89% for subgenus *Leptostemonum* (with *S. wendlandii* outside the subgenus).

Chloroplast data set combined (Figure 2) - The partition homogeneity test to evaluate the congruence between the *trn*L-*trn*F + *trn*L intron fragment (combined) and *trn*S-*trn*G fragment partitions detected a insignificant conflict between these two datasets, which is indicated by the *p* value obtained (0.280). The *trn*L-*trn*F + *trn*L intron + *trn*S-*trn*G were combined and included 2,137 characters for 52 taxa (individuals having the total sequences for all fragments of the chloroplast were used) (Table 1). For 2,137 characters 221 were parsimony-informative. The general frequencies of the four nucleotides A, C, G, and T were, respectively, 0.35, 0.18, 0.15, and 0.32 (GC = 33%). The g1 statistic for the trees was of -0.177. The phylogenetic analysis resulted in 865 steps, with CI = 0.72 and RI = 0.85 (Table 2). The 50% majority rule consensus tree presents two large groups (Figure 2). The first group is composed by *Solanum* section *Torva* (BS 99) and *S. robustum*, *S. jamaicense* plus *S. melongena* as sister species of the section *Torva*. The species of the section *Torva* were divided into two subgroups with the first one (BS 92) formed by *S. variabile* and *S. paniculatum*, being adjacent to the intermediate morphology species. The second subgroup is formed by *S. guaraniticum* related with the

intermediate morphology species (BS 85). *Solanum torvum* maintained its position as the most basal species of the section *Torva*. The second group is composed by two subgroups, one formed by the section *Acanthophora* (BS 100), and other by the section *Lasiocarpa* (BS 100), this wide group has *S. stenandrum* of the section *Acanthophora* as the most basal species. We observed a strong relationship between the sections *Lasiocarpa* (monophyletic) and *Acanthophora* (not monophyletic) with BS of 97. *S. sisymbriifolium* and *S. stagnale* are inside the large group formed by species of the subgenus *Leptostemonum*, while *S. wendlandii*, a spiny species, is outside the ingroup and is related to non-spiny species. The evolutive model selected for the Bayesian analyses was TVM+I+G, with gamma shape parameters estimated of 0.8395. The Bayesian tree topology showed be similar to the parsimony analyses except for *S. sisymbriifolium* and *S. robustum* that came up once as a sister species of the section *Torva* (Bayesian and parsimony analyses respectively) and *S. aculeatissimum* that is placed in an ambiguous position related the section *Acanthophora* (Bayesian and parsimony analyses respectively). The distance analysis showed less resolution in the tree than the Parsimony and Bayesian analyses. In the former analysis *Solanum* section *Torva* and the subgenus *Leptostemonum* are also monophyletic (BS 92 and 74, respectively) with *S. wendlandii* outside the subgenus.

All data sets combined - The partition homogeneity test to evaluate the congruence among the chloroplast and ITS data partitions detected a significant conflict between these two datasets, which is indicated by the *p* value obtained (0.001). Even so it was decided to combine the two partitions, since interesting results can be obtained from simultaneous analysis even if the constituent data partitions display heterogeneity, by generating more

robust trees. Total chloroplast data plus ITS were combined and included 2,864 characters for 45 taxa (Table 1). Of these characters, 414 were parsimony informative (PI). The general frequencies of the four nucleotides A, C, G, and T were, respectively, 0.30, 0.23, 0.19, and 0.28 (GC = 42%). The g1 statistic for the trees was of -0.316. In the maximum-parsimony analyses, the heuristic search resulted in 18 most-parsimonious trees of 1,737 evolutionary steps with index of consistency (CI) of 0.64 and retention index (RI) of 0.79 (Table 2). The 50% majority rule consensus tree presents two large groups, being 39 clades indicated (Figure 3) with their respectively support (Table 3 (PBS support) and Table 4). The first group is represented by *Solanum* section *Torva* (Clade 13 - Figure 3, Table 3 and 4) plus *S. jamaicense*, *S. melongena* and *S. sisymbriifolium* as sister species of the section *Torva* (Clade 16 - Figure 3 Table 3 and 4). The section *Torva* was divided into two subgroups each supported by BS 81. One subgroup is composed by the morphology intermediate species and *S. paniculatum* that is more adjacent to *S. variabile* (Clade 6 - Figure 3, Table 3 and 4). The other subgroup is composed by the morphology intermediate species and *S. guaraniticum* (Clade 11, Figure 3, Table 3 and 4). *S. torvum* showed a basal position within the section *Torva*. *Solanum wendlandii* is outside of the group formed by the subgenus *Leptostemonum* (Clade 39 - Figure 3, Table 3 and 4). The evolutive model selected for the Bayesian analyses was GTR+I+G, with gamma shape parameters estimated of 0.6763. Bayesian and distance analysis showed a similar tree topology to that of the parsimony analysis. These analyses differed from that of the parsimony analysis by the position of *S. robustum*, *S. stagnale* and *S. stenandrum*, which are adjacent to the section *Torva* (BS 99), and of *S. sisymbriifolium* that is found not related from the section *Torva*. The subgenus *Leptostemonum* presents also strong support in Bayesian and distance analyses (Clade 39 - Figure 3, Table 3).

To assess the relative contribution of each fragment in our combined analysis the PBS scores for each fragment on each node were calculated. This test confirmed that the chloroplast region contributes more to the results than the ITS regions (Table 4) once it provided almost three times more information than ITS and contributed much more to the formation of clades in the combined analysis (Figure 3 and Table 4). Similarly, the *trn*S-*trn*G intergenic spacer proved be more informative than the combination of the intron *trn*L plus the *trn*L-*trn*F spacer. Furthermore, the breakdown of the PBS values also indicated substantial conflict between the chloroplast and ITS data partitions, because 15 of the 39 nodes resolved in the combined analysis had conflicting PBS values. This pattern may have been established because of the high homoplastic index in the ITS regions.

Discussion

The section *Torva* - The analyses realized in this study suggest that *Solanum* section *Torva* is monophyletic and it is in agreement with Levin et al. (2006). The sections *Lasiocarpa* and *Acanthophora* are respectively monophyletic and paraphyletic as it were proposed by Bohs (2004) and Levin et al. (2005). We had the same results when species from section *Torva* and other from section *Acanthophora* (specifically those from Southern Brazil) were included. Section *Acanthophora* is not monophyletic due to the inclusion of *S. stenandrum* (in all analyzed matrixes) plus *S. agrarium* (based on the analyses using ITS and *trn*S-*trn*G data). This agrees with the work by Levin et al. (2005) although the section *Acanthophora* showed strongly related with the section *Torva* by its morphological characters (Mentz, personal

communication). Section *Acanthophora* proved be strongly related to the section *Lasiocarpa* in this work, in agreement with the data provided by Bohs (2004) and Levin et al. (2005) which indicated that they are sister taxa.

Solanum section *Torva* is divided into two internal groups in all analyses, with the basal species being *S. torvum*. The groups of the section *Torva* clearly form a strongly relationship among the intermediate morphology species and the possible progenitors (*S. paniculatum* and *S. guaraniticum*). This relation is not evident in the analyses performed from the ITS data, where no particular relationship between *S. paniculatum* and the intermediate morphology species was found. This finding, however, cannot be taken as conclusive because of the high homoplastic index (HI) shown for ITS marker and by concerted evolution, responsible by homogenization of DNA sequence *en tandem* of ribosomal DNA. In the work realized by Chase et al. (2003), concerning the origin of hybrids in *Nicotiana* (Solanaceae), the authors commented that ITS is not a generally reliable tool for detection of hybrids, once that the homogenization may be directional accordant with a paternal ITS allele (e.g., *N. tabacum*) or with a maternal ITS allele (e.g., *N. rustica*). However, in the present work it may be suggested that the homogenization of ITS was in direction of the paternal allele, and it would be indicate *S. guaraniticum* as the paternal progenitor because all representatives of intermediate morphology species are close to *S. guaraniticum* from ITS analysis and related to *S. paniculatum* based on plastidial analysis (that is supposed inherited by maternal origin), Figures 1 and 2.

The section *Torva* of *Solanum* proved be very close to the species: *S. melongena*, *S. jamaicense* and *S. sisymbriifolium* (species from other sections of the subgenus *Leptostemonum*), in most of the performed analyses suggesting that these species can be sister

of section *Torva*. This relationship also is showed in the work of Levin et al. (2005) where the species *S. jamaicense*, *S. torvum*, *S. melongena* and *S. sisymbriifolium* formed a group supported by 100 % of bootstrap.

The monophyly of *Solanum* section *Torva* showed in this study may not be applicable to the section *Torva* worldwide because our sample of non-Brazilian plants was small but is certainly applicable and correct for the plants which grow in the southern region of Brazil. The samples of section *Torva* used here represent 60% of the Southern Brazil species, 50% of the brazilian species, but only 13% of the Southern American species and 10% of total species of section *Torva* in the world (according to Nee, 1999).

Subgenus *Leptostemonum* – The subgenus *Leptostemonum* was found non-monophyletic in all performed analyses in this study. *Solanum wendlandii* is placed outside the group formed by the spiny species and adjacent to the non-spiny species of the outgroup. In the work realized by Bohs (2004), all spiny taxa of *Solanum* with exception of *S. wendlandii* formed a monophyletic group supported by 100% of bootstrap in all analyses. The author further reported that *S. wendlandii* was also excluded from the *Leptostemonum* clade in previous molecular analyses realized by Bohs and Olmstead (1997, 1999, 2001) and Levin et al. (2005). In the present work we also observed that *S. wendlandii* is very well supported outside the group formed by the species of the subgenus *Leptostemonum*, where it has been traditionally included. *Solanum wendlandii* display simple and glandular trichomes, different from the *Leptostemonum* species which show simple and stellate trichomes. The theory that this species should be included in *Leptostemonum* is based only on the presence of the spines (Whalen, 1984). Child (1983, 1990) emphasizing the lack of stellate hairs, transferred these plants from the *Leptostemonum* to a new position adjacent to the subgenus Potato (G.Don) D'Arcy. Nee

(1999), included *S. wendlandii* within the subgenus *Leptostemonum*, but also discussed its proximity with the *Dulcamara* and *Petota* sections (based on the morphology of flowers, leaves and inflorescence). The phylogenetic and morphological evidences discussed here permit us to propose the exclusion of *S. wendlandii* from the subgenus *Leptostemonum* which, consequently, becomes a monophyletic subgenus. It is also evident in the recent work of Levin et al. (2006) where it was proposed the exclusion of *S. wendlandii* group (including *S. wendlandii* and *S. refractum* Hook. & Arn. species) and *S. nemorense* Dunal group (*S. hoehnei* C.V. Morton, *S. reptans* Bunbury and *S. nemorense*) of *Solanum* subgenus *Leptostemonum*. The authors commented that these groups are formed by species with prickles but lack stellate hairs.

Comparison among markers

A partition homogeneity test (PHT) realized from a maximum parsimonious tree obtained from four fragments, showed significant incongruence between the chloroplast and ITS fragments. This incongruence can be observed in the tree topology based on the plastid DNA only, and in the tree derived from the combined chloroplast and ITS data which differ in few clades. The plastid DNA presents a greater degree of consistency when compared to the analyses based on the ITS region (Table 4) and showed almost three times the partition Bremer support (PBS) scores as compared to the ITS region (Table 4). This discrepancy may be caused by the different mode of inheritance of the markers (chloroplast = maternal inheritance, nuclear = biparental); by the high rates of homoplasy found in ITS, (due mainly to the rapid evolution of the region as compared to the other regions of the plastid genome); and

also to the greater number of characters obtained with the cpDNA data (2,137 bp) compared to the data from the ITS region (727 bp) used for the analysis of the four combined fragments.

The nuclear region (ITS) presented a high degree of phylogenetically informative characters (31.4%) in comparison with other markers. This evidence is also clear in the Hillis test, in which the ITS data matrix shows an asymmetrical distribution of the tree lengths to the right (phylogenetic tree with the lowest number of steps) with a smaller interval to the left (g1 = -0.44) than the other matrixes. However, the consistency index was low for ITS in agreement with the work by Levin et al. (2005, 2006). The analysis based only on ITS with the greater taxon sampling from *Solanum* section *Lasiocarpa* plus species from section *Torva* evidenced that section *Lasiocarpa* is monophyletic as in agreement with the data obtained by Bohs (2004). In the latter analysis we observed an approximation of the so-called intermediate species with *S. guaraniticum* - this difference may be due to the biparental inheritance, or perhaps to the high homoplastic index of the fragment, which may be concealing more precise information about the degree of relationship. The analyses realized with the ITS matrix allowed the separation among the sections *Torva, Acanthophora* and *Lasiocarpa,* and subgenus *Leptostemonum*, but showed variation in some taxa of section *Acanthophora.* Besides this variation it showed not invalidate its use as phylogenetic marker, since all the individuals from the same species but one (*S. aculeatissimum*) form a clade with high support. Therefore, the variation of ITS do not pass the species barrier and showed that it is an efficient phylogenetic marker. In addition, the problem concerning the monophyly of *S. aculeatissimum* was also showed when the plastid regions were used as characters.

The partition homogeneity test (PHT) realized with the cpDNA matrix showed high congruency among the chloroplast fragments. This was also found in the PBS scores where

conflicts were observed in only nine of the thirty-nine clades obtained with the consensus tree representing the four combined fragments when we considered only the chloroplast data (Table 4).

The phylogenetic tree obtained from cpDNA presented a greater number of informative characters in comparison with the individual analyses of the intron *trn*L + the *trn*L-*trn*F spacer fragments and the *trn*S-*trn*G spacer (Table 2). Amongst the individual cpDNA analyses the fragment which showed the greatest number of information corresponds to the intron *trn*L + the *trn*L-*trn*F spacer. These results contradict the work by Shaw et al. (2005), where they found that the *trn*S-*trn*G spacer has a higher PI index than the co-amplified region of the intron *trn*L + spacer *trn*L-*trn*F.

However, using *Solanum* species and verifying each fragment individually, we found greater variability in the intron *trn*L followed by the *trn*L-*trn*F spacer than that of the spacer *trn*S-*trn*G.

Many works were realized using cpDNA to infer phylogenies of Solanaceae, including the genus *Solanum* in different taxonomic levels (Olmstead and Palmer, 1991; Bohs and Olmstead, 1999; Olmstead et al., 1999; Bohs, 2004; Clarkson et al., 2004; Levin et al. 2005 and 2006). Generally, the plastid genome typifies conservative evolution and consequently displays a smaller number of phylogenetically informative characters when compared to nuclear genome. In the present work, however, the chloroplast regions exhibit a relatively high degree of divergence, which helps to elucidate the relationships among the taxa of the genus *Solanum.* The chloroplast analyses permit the formation of highly supported clades which showed the relationship among individuals of the same species and taxa of the same section, and furthermore, draw a distinction among the species armed with stellate hairs and the other

species not belonging to the subgenus *Leptostemonum*. Among the analyses realized, only in those of the plastid fragments did we find a height consistency index for the isolated analysis of fragments corresponding to the intron *trn*L + the spacer *trn*L-*trn*F combined, perhaps because a greater number of these taxa were included in the analysis (76) in comparison to the other fragments. However, the tree showing a greater resolution is that which combines all the chloroplast fragments in only one matrix, showing that in addition of the taxon sampling, the number of characters contributes to a better phylogenetic inference.

Conclusion

In this work, the monophyly of the sections *Torva* and *Lasiocarpa*, and the paraphyly of the section *Acanthophora* are showed and the latter is shown to be a sister section of the section *Lasiocarpa*. Evidence for the considerable consistency of the interaction of the intermediate species with both of its so-called progenitor species is showed, reinforcing the suggestion that an intermediate species to both *S. guaraniticum* and *S. paniculatum* exist. The cpDNA fragments were found to be more efficient in clarifying the phylogenetic relationships between the taxa of the *Solanum* genus than those of ITS because the latter presented an elevated percentage of homoplastic characters. The removal of *S. wendlandii* from its present position in the *Leptostemonum* subgenus should be proposed, as this would result in the subgenus being accepted as monophyletic.

The phylogenetic relationship of *Solanum* section *Torva* clarified in the present study may assist in the search for alternative species from which important secondary metabolites

may be obtained for pharmacological production, as well as contributing to sustainable utilization, and better exploitation of the species.

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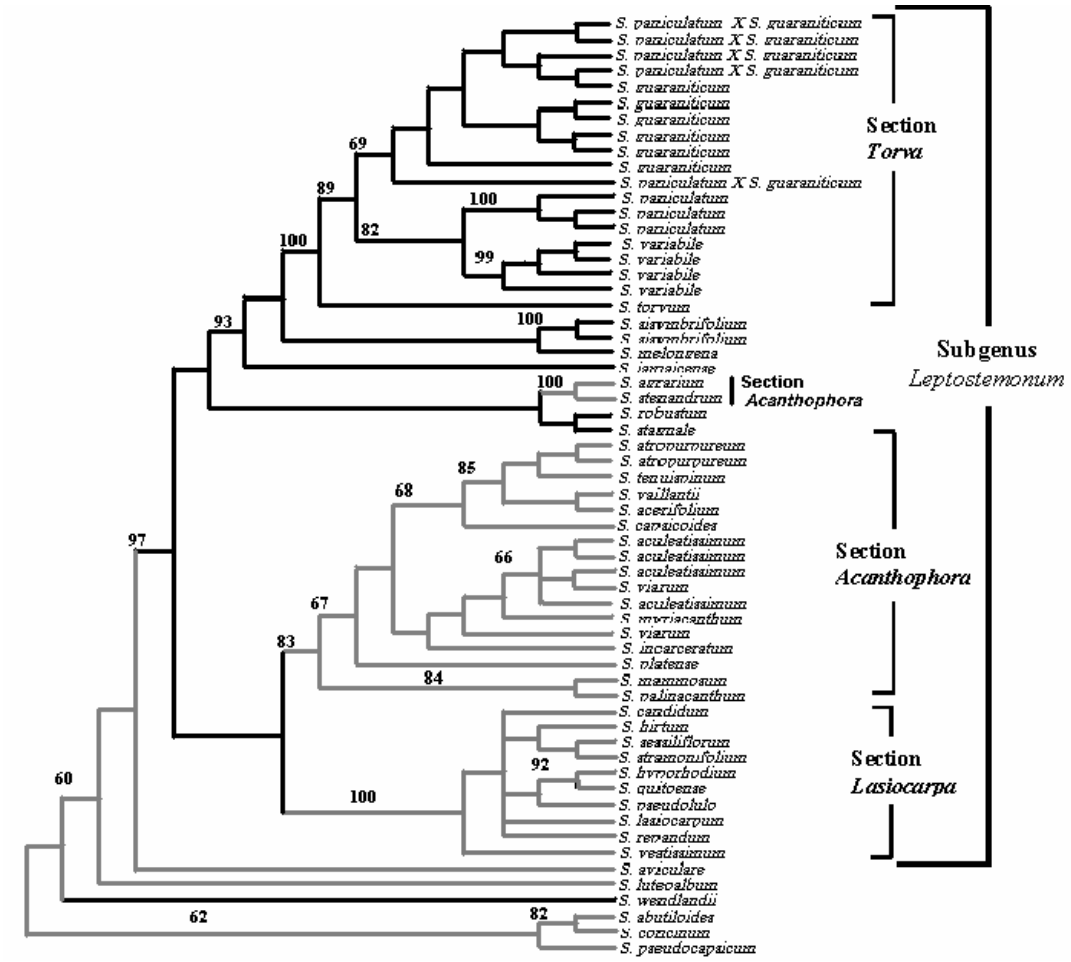

Figure1: Majority consensus (50%) tree of the 20,000 (Maxtrees) most parsimonious trees from ITS data. Numbers above branches correspond to bootstrap support values (when higher than 50%) based on 1,000 replicates.

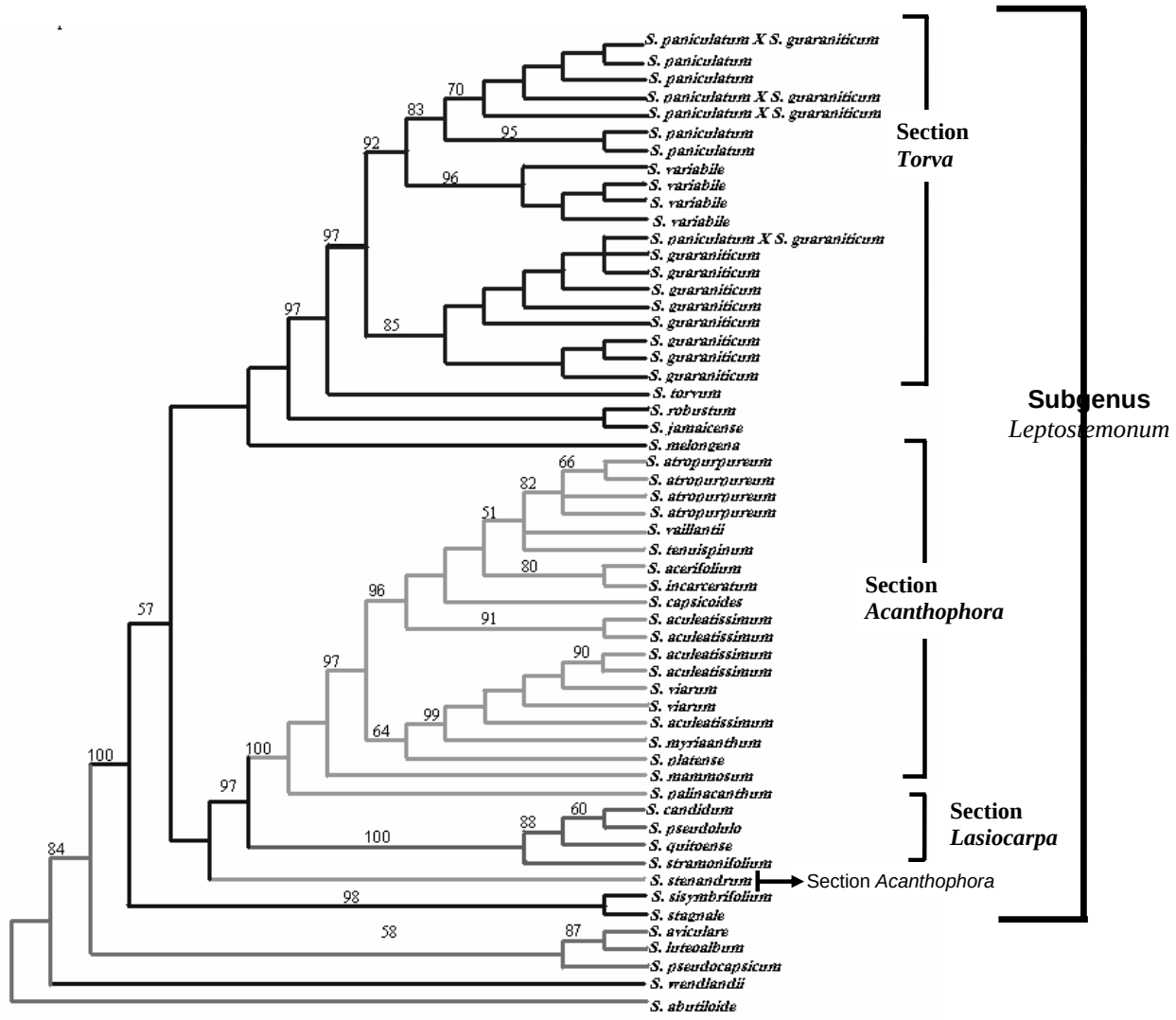

Figure 2: Majority consensus (50%) tree of the 20,000 (Maxtrees) most parsimonious trees from the plastid data. Numbers above branches correspond to bootstrap support values (when higher than 50%) based on 1,000 replicates.

Figura 3: Majority consensus (50%) of the 20,000 (Maxtrees) most parsimonious trees from four fragments combined data. In face of the internal branches there is an arbitrarily defined number representing the clade that follows and which can be used to interpret Tables 3 and 4.

Table 1:

Table 1: List of *Solanum* species from section *Torva* (A), *Acanthophora* (B), *Lasiocarpa* (C) and other species of the subgenus *Leptostemonum* (D) plus species used as outgroup (no spiny species) (E), used in this study; with their respective voucher, location and GenBank accession numbers for regions ITS, intron *trn*L plus *trn*L-*trn*F spacer, *trn*S-*trn*G spacer. The asterisks correspond to the fragments used for each taxon and N to the missing data. Where there is no data about sampling places the sequences were get from GenBank.

A)

	Taxa	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intron *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	Sampling Places
	Solanum* section *Torva					
1	*Solanum paniculatum* X *Solanum guaraniticum*	L. A. Mentz, T.T Souza-Chies and R.B. Miz, 301.	*	*	*	RS, Porto Alegre
2	*Solanum paniculatum* X *Solanum guaraniticum*	L. A. Mentz, T. T. Souza-Chies and R. B. Miz, 302.	*	*	*	RS, Porto Alegre
3	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 309 - ICN	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
4	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva,	**N**	*	*	RS, Viamão, State Park of Itapuã

		G.S. Vendruscolo, 305- ICN				
5	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S .Vendruscolo, R.B. Miz, 311 - ICN	*	*	**N**	RS, Carazinho
6	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares , R.B. Miz, 344 – ICN.	*	*	*	RS, Venâncio Aires
7	*Solanum guaraniticum* A. St.-Hil.	T. T. Souza-Chies, 221.	*	*	*	RS, Antônio Prado, Amarilho Road,
8	*Solanum guaraniticum* A. St.-Hil.	T.T. Souza-Chies, 222	N	*	**N**	RS, Monte Bonito, district of Pelotas
9	*Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares , R.B. Miz, 349.	*	*	*	RS, Santa Cruz
10	*Solanum guaraniticum* A. St.-Hil. (spiny leave)	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B. Miz, 336- ICN.	N	*	*	RS, Fontoura Xavier, BR 386, km 276
11	*Solanum guaraniticum* A. St.-Hil. (spiny low stem)	L.A. Mentz, E.L.C. Soares , R.B. Miz, 345a.	N	*	*	RS, Santa Cruz
12	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares , R.B. Miz, 345b.	*	*	**N**	RS, Santa Cruz
13	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares , R.B. Miz, 345c.	N	*	*	RS, Santa Cruz
14	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares , R.B. Miz, 339.	*	*	**N**	RS, Coronel Barros - Stop Lajeado do Tigre – BR285 Km 476
15	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B. Miz, 333.	*	*	*	RS, Santo Ângelo
16	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares , R.B. Miz, 348.	*	*	*	RS, Santa Cruz
17	*Solanum paniculatum L.*	R.B. Miz, 101.	N	*	*	RS, Porto Alegre
18	*Solanum paniculatum L.*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva,	**N**	*	*	RS, Viamão, State Park of Itapuã

		G.S. Vendruscolo, 304-ICN.				
19	*Solanum paniculatum L.*	M. F. Agra, K. Nurit and I. Basílio, 6311.	*	*	**N**	Paraíba, Municipal district João Pessoa, Campus of the Federal University of the Paraíba
20	*Solanum paniculatum L.*	L.A. Mentz, E.L.C. Soares, R. B. Miz, 341-ICN.	*	*	*	RS, Triunfo
21	*Solanum paniculatum L.*	L.A. Mentz, E.L.C. Soares, R.B. Miz, 340-ICN.	*	*	*	RS, Triunfo
22	*Solanum variabile* Mart.	L. Essi Li 301	*	*	*	RS/SC, Cambará do Sul. "Serra do Faxinal"
23	*Solanum variabile* Mart.	L. Essi Li302	*	*	*	SC, Lauro Muller
24	*Solanum variabile* Mart.	L. Essi Li304	*	*	*	SC, Bom Jardim da Serra. Mountain of "Rio do Rastro"
25	*Solanum variabile* Mart.	L. Essi Li319	N	*	*	SC, Urubici
26	*Solanum variabile* Mart.	L. Essi Li320	*	N	**N**	SC, Urubici
27	*Solanum torvum Sw.*	_	* (AF244729)	* (AY266246)	* (AY555478)	GenBank

B)

	Taxa	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intron *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	Sampling Places
	Solanum* section *Acanthophora					
1	*Solanum atropurpureum* Schrank	L.A. Mentz, E.L.C. Soares, R. B. Miz, 343-ICN.	*	*	*	RS, Santa Cruz
	Solanum atropurpureum Schrank	.A. Mentz, E.L.C. Soares, R. B. Miz, 342- ICN.	**N**	*	*	RS, Santa Cruz
3	*Solanum atropurpureum* Schrank	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 325a. ICN.	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
4	*Solanum atropurpureum* Schrank	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz. 325b- ICN.	**N**	*	**N**	RS, Passo Fundo, FarmSementes and Cabanhas Butiá
5	*Solanum atropurpureum* Schrank	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 317- ICN.	**N**	*	**N**	RS, Ijuí, Estrada Ijuí-Catuipe
6	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, R. B. Miz, 347-ICN.	**N**	*	*	RS, Santa Cruz
7	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B. Miz, 319- ICN.	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
8	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S.	**N**	*	**N**	RS, Passo Fundo, Farm Sementes and Cabanhas

		Vendruscolo, R.B. Miz, 334- ICN.				Butiá
9	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 313- ICN.	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
10	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 326a- ICN	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
11	*Solanum aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 326b- ICN	*	*	**N**	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
12	*Solanum viarum* Dunal	L.A. Mentz, E.L.C. Soares, R.B. Miz, 346- ICN.	*	*	*	RS, Santa Cruz
13	*Solanum vaillantii* Dunal	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 310- ICN.	*	*	*	RS, Passo Fundo, Farm Sementes and Cabanhas Butiá
14	*Solanum acerifolium* Dunal	_	* (AY561261)	* (AY266249)	* (AY555454)	GenBank
15	*Solanum aculeatissimum* Jacq.	_	* (AY561262)	* (AY559236)	* (AY555455)	GenBank
16	*Solanum agrarium* Sedtn.	_	* (AY561263)	**N**	* (AY555456)	GenBank
17	*Solanum atropurpureum* Schrank	_	* (AY561264)	* (AY559237)	* (AY555457)	GenBank
18	*Solanum capsicoides* All.	_	* (AY561265)	* (AY266251)	* (AY555460)	GenBank
19	*Solanum incarceratum* Ruiz & Pavon	_	* (AY561266)	* (AY559239)	* (AY555461)	GenBank
20	*Solanum mammosum* L.	_	* (AF244721)	* (AY559240)	* (AY555464)	GenBank
21	*Solanum myriacanthum* Dunal	_	* (AY561267)	* (AY559240)	* (AY555466)	GenBank

22	*Solanum palinacanthum* Dunal	_	* (AY561268)	* (AY266233)	* (AY555467)	GenBank
23	*Solanum platense* Diekman	_	* (AY561269)	* (AY559241)	* (AY555468)	GenBank
24	*Solanum stenandrum* Sendtn.	_	* (AY561273)	* (AY559242)	* (AY555475)	GenBank
25	*Solanum tenuispinum* Rusby	_	* (AY561274)	* (AY266245)	* (AY555477)	GenBank
26	*Solanum vaillantii* Dunal	_	**N**	**N**	* (AY555479)	GenBank
27	*Solanum viarum* Dunal	_	* (AY561275)	* (AY559243)	* (AY555480)	GenBank

C)

	Taxa	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intron *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	Sampling Places
	Solanum* section *Lasiocarpa					
1	*Solanum candidum* Lindl.	_	* (AF244722)	* (AY266250)	* (AY555459)	GenBank
2	*Solanum felinum* Whalen	_	**N**	* (AY266252)	**N**	GenBank
3	*Solanum hirtum* Vahl	_	* (AY263458)	* (AY266254)	**N**	GenBank
4	*Solanum hyporhodium* A. Braun & Bouché	_	* (AY263461)	* (AY266238)	**N**	GenBank
5	*Solanum lasiocarpum* Dunal	_	* (AY263457)	* (AY266256)	**N**	GenBank
6	*Solanum pectinatum* Dunal	_	**N**	* (AY266230)	**N**	GenBank
7	*Solanum pseudolulo* Heiser	_	* (AY263459)	* (AY266242)	* (AY555470)	GenBank
8	*Solanum quitoense* Lam.	_	* (AY263460)	* (AY266228)	* (AY555471)	GenBank
9	*Solanum repandum* G. Forst.	_	* (AY263466)	* (AY266234)	**N**	GenBank
10	*Solanum sessiliflorum* Dunal	_	* (AY263455)	* (AY266260)	**N**	GenBank
11	*Solanum stramoniifolium* Jacq.	_	* (AY263465)	* (AY266244)	* (AY555476)	GenBank
12	*Solanum vestissimum* Dunal	_	* (AY263467)	* (AY266264)	**N**	GenBank

D)

	Taxa	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intron *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	Sampling Places
	Outgroups in subgenus *Leptostemonum*					
1	*Solanum sisymbriifolium* Lam.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 351-ICN.	*	*	N	RS, Porto Alegre – Campus of Vale- UFRGS
2	*Solanum melongena* L.	_	* (AF244726)	* (AY266240)	* (AY555465)	GenBank
3	*Solanum robustum* Wendl.	_	* (AY561270)	* (AY266259)	* (AY555472)	GenBank
4	*Solanum sisymbriifolium* Lam	_	* (AY561271)	* (AY266235)	* (AY555473)	GenBank
5	*Solanum stagnale* Moric.	_	* (AY561272)	* (AY266262)	* (AY555474)	GenBank
6	*Solanum wendlandii* Hook. f.	_	* (AF244731)	* (AY266248)	* (AY555481)	GenBank
7	*Solanum jamaicense* Mill.	_	* (AF244724)	* (AY266239)	* (AY555472)	GenBank

E)

	Taxa	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intro *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	Sampling Places
	Outgroups outside subgenus *Leptostemonum* (non—spiny)					
1	*Solanum mauritianum* Scop.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 350-ICN.	**N**	*	**N**	RS, Porto Alegre – Campus of Vale - UFRGS
2	*Solanum abutiloides* (Griseb.) Bitter & Líllo	_	* (AF244716)	* (AY266236)	* (AY555453)	GenBank
3	*Solanum aviculare* Forst. F.	_	* (AF244719)	* (AY559238)	* (AY555458)	GenBank
4	*Solanum luteoalbum* Pers.	_	* (AF244715)	* (AY266257)	* (AY555463)	GenBank
5	*Solanum pseudocapsicum* L.	_	* (AF244720)	* (AY266241)	* (AY555469)	Genbank
6	*Solanum dulcamara* L.	_	**N**	* (AY266231)	**N**	GenBank
7	*Solanum concinnum* Schott ex Sendtn.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, 388-ICN.	*	**N**	**N**	RS, Viamão, State Park of Itapuã

Table 2: Comparison among the nuclear and plastid data from *Solanum*.

[U2] Comentário:

Statistic parameters	ITS	*trn*S-*trn*G	Intron *trn*L + *trn*L-*trn*F	chloroplast data matrix	combined matrix
Taxa number used	60	58	76	56	45
Aligned length	727bp	868bp	1270bp	2137bp	2864bp
Trees length	1079	319	640	865	1737
Variable sites	363bp	206bp	387bp	523bp	832bp
PI sites	228bp	87bp	216bp	221bp	414bp
CI (RC);	0.55 (0.44)	0.72 (0.62)	0.74 (0.67)	0.72 (0.62)	0.64 (0.51)
RI	0.80	0.86	0.89	0.85	0.79

Note: PI= Parsimony – informative; CI = consistency index (RC= rescaled CI); RI= retension index.

Table 3: Bootstrap values presented for each clade shown in Figure 3 (NJ and MP) and consensus values in Bayesian analysis by the constructed tree using different phylogenetic reconstruction methods.

Clade	D (NJ)	MP	Bayesiana
1	58	59	92
2	100	100	100
3	99	100	100
4	71	98	87
5	52	95	100
6	-	81	99
7	-	-	-
8	88	65	83
9	-	83	83
10	58	75	87
11	87	81	100
12	91	100	100
13	99	100	100
14	-	53	-
15	73	-	100
16	-	97	100
17	-	-	-
18	-	53	-
19	-	95	93
20	92	-	-
21	56	73	99
22	91	86	100
23	-	62	93
24	-	69	-

25	69	95	100
26	65	86	100
27	-	-	98
28	96	74	100
29	100	93	100
30	73	66	96
31	99	100	100
32	-	-	-
33	80	97	98
34	100	100	100
35	91	95	100
36	-	-	-
37	-	-	-
38	-	-	-
39	97	100	100

Table 4: Partition Bremer support (PBS) scores across the simultaneous analysis of Parsimony consensus tree for each fragment (intron *trn*L plus *trn*L-*trn*F, *trn*S-*trn*G and ITS) partition in each clade presented in Fig 3.

Clade	**Intron *trn*L + *trn*L-*trn*F**	***trn*S-*trn*G**	**ITS region**	**Decay index**
1	-1	0	2	1
2	3.07	0.63	3.31	7
3	-1.06	0.50	5.56	5
4	-1.06	0.50	1.56	1
5	-0.24	-0.05	1.29	1
6	0	0.50	-0.50	0
7	0.07	0	-0.07	0
8	-0.14	0	1.14	1
9	0	0	0	0
10	-0.06	-0.50	2.56	2
11	-0.86	2.50	0.36	2
12	2.14	-0.30	1.16	3
13	2.94	1	9.06	13
14	-0.24	0.55	-0.31	0
15	-0.73	2.30	-0.61	1
16	2.44	1.50	-1.94	2
17	0	0	0	0
18	0	0	0	0
19	1	0	0	1
20	0	0	0	0
21	0	0	2	2
22	0.33	1	3.67	5
23	0	0	1	1

24	0	0	0	0
25	0	0	5	5
26	1	1	3	5
27	0	0	2	2
28	3	1	0	4
29	1	1	3	5
30	-1.42	-0.21	3.63	2
31	3.90	2.98	0.12	7
32	0	0	0	0
33	1.63	-0.63	1	2
34	2.94	2.45	10.60	16
35	3.94	0	-1.94	2
36	1.94	1	-2.94	0
37	1.94	1	-2.94	0
38	1.94	1	-2.94	0
39	7.54	4.50	5.96	18
Total PBS	35.95	25.22	54.79	-
Min steps	312	160	605	-
Total PBS/Min steps[a]	0.11	0.15	0.09	-

[a] PBS values summed across the tree and standardized by the minimum number of steps for each partition.

4.- Artigo 2

Phylogeny and genetic variation of species of

***Solanum* section *Torva* (Solanaceae) from Southern Brazil**

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Phylogeny and genetic variation of species of

***Solanum* section *Torva* (Solanaceae) from Southern Brazil**

Rogéria B. Miz [a], Tatiana T. Souza - Chies [a, b]

[a] Universidade Federal do Rio Grande do Sul, Programa de Pós Graduação em Genética e Biologia Molecular, Av. Bento Gonçalves, 9500. Caixa Postal 15053, CEP: 91501-970, Porto Alegre, RS, Brazil. Tel.:+55 51 3316-9830, Fax: + 55 51 3316-7311

[b] Universidade Federal do rio Grande do Sul, Departamento de Botânica, Av. Bento Gonçalves, 9500. Caixa Postal 15053, CEP: 91501-970, Porto Alegre, RS, Brazil. Tel.:+55 51 3316-7569, Fax: + 55 51 3316-7686

Corresponding author: **Dra. Tatiana T. Souza-Chies**

Address for correspondence.

Departamento de Botânica, UFRGS

Prédio 43433

CEP: 91501-970, Porto Alegre, RS, Brazil

Tel.: 55 51 3316-7569

Fax: 55 51 3316-7686

E-mail: tatiana.chies@ufrgs.br

Abstract

Worldwide *Solanum* subgenus *Leptostemonum* section *Torva* comprises approximately 40 species. Five of them are found in southern Brazil. Some of those species contain active substances which are useful in the treatment of gastrointestinal diseases and ulcerations. However, these plants present taxonomic problems concerning their circumscription, and their evolutionary relationships are hard to understand. In addition, there is a group of plants presenting an intermediate morphology between *S. paniculatum* x *S. guaraniticum*. In this study, three marker types were used (nuclear, plastid and ISSR) to investigate about the phylogenetic relationships and to evaluate the variation among the taxa from section *Torva.* The ISSR markers showed a high degree of polymorphism with great variation identified both inter and intra specific levels for the studied species. All the phylogenetic analysis indicated that the intermediate morphology (*S. paniculatum* x *S. guaraniticum*) species is related to their supposed progenitors *S. paniculatum* and *S. guaraniticum.* Furthermore, we observed that *S. guaraniticum/S. bonariense*, and *S. adspersum/S. tabacifolium* are very close. The southern Brazilian species of *Solanum* section *Torva* can be considered as monophyletic.

Key Words: Solanaceae; *Solanum* section *Torva*; ITS; intron *trn*L plus *trn*L-*trn*F; *trn*S-*trn*G; phylogenetic analysis; ISSR marker; genetic variation.

Introduction

Solanum L. presents some species that are of special interest because of their active elements, which have pharmacological properties of great importance in medicine and to produce therapeutic drugs. Among the species under investigation *S. tuberosum* L., *S. stramonifolium* Dunal, *S. siparunoides* Ewan, *S. dulcamara* L., *S. nigrum* L. and *S. indicum* L. (Frusciante *et al*., 2000; Syu *et al*., 2001; Lee *et al*., 2004) are some examples.

Notwithstanding the importance of this genus for the pharmaceutical industry, many other lesser known species are intensely used in folk medicine. This intense use could create a risk of extinction of innumerable native plants resulting in ecological disturbances and the disappearance of valuable germ plasm of which pharmaceutical and chemical potential has not yet been established. To preserve these species is indispensable to know about the biodiversity. Knowledge of the taxonomy and genetic diversity of these species is fundamental for sustainable utilization of these important natural resources. One of the *Solanum* groups which requires taxonomic study to improve understanding of its species, and whose pharmaceutical potential is of outstanding interest, is the section *Torva* (Nees, 1984). The species of *Solanum* section *Torva* contain active substances for the treatment of gastrointestinal diseases and ulcerations (Mesia-Vela *et al*., 2002; Antonio *et al*., 2004).

Section *Torva* belongs to the subgenus *Leptostemonum* (Dunal) Bitter of *Solanum* (Solanaceae), and is composed of more than 40 species (Nee, 1999) distributed worldwide (its dispersion centre is in the Americas, and some species are found in África, Ásia, New Guinea and in the Pacific). In southern Brazil, five species are known: *Solanum paniculatum* L., *Solanum guaraniticum* A. St.-Hil., *Solanum adspersum* Witasek, *Solanum variabile* Mart. and *Solanum tabacifolium* Dunal.

Solanum paniculatum (known as the real "*jurubeba*") is a perennial shrub species, erect and few branched, armed on stems and branches with curved aculeous widened and flattened at the base. Leaves bicolour, abaxially covered by dense pubescence with sessile stellate hairs. Calyx covered by dense pubescence, lobes wide with a narrow tip. Corolla white or pale violet, lobes divided into the half. Fruits are pendent. Its reproduction is made by seeds. This species is most often found in the eastern cost of Brazil, from the state of Rio Grande do Norte in the extreme North to the southern state of Rio Grande do Sul. Sometimes it is also found as cultivated crop or as a ruderal. According to Mentz & Oliveira (2004), the populations now found in Rio Grande do Sul have been recently introduced.

Solanum guaraniticum (one of the false "*jurubeba*") is a perennial shrub species, erect and few branched, armed on stems and branches with straight acicular aculeous, of different lengths, occasionally slightly widened at the base. Populations can be found with no prickles or nearly unarmed. Leaves almost concolor, covered with stellate trichomes, in general having a tiny pedicel. Calyx with strait-triangular lobes, nearly glabrous or covered by stellate trichomes. Corolla white or very occasionally slightly bluish, divided above the middle. Fruits erect. Generally with reproductive roots, but it also reproduces from seeds. This species presents ruderal behaviour and is found also in Paraguay and Argentina - in Brazil is distributed from the state of Minas Gerais to the state of Rio Grande do Sul in the extreme South Region. In the city of Porto Alegre and environs, some populations can not be identified exactly as either *S. paniculatum*, or *S.guaraniticum*, because they showed an intermediate morphology between these two species (Mentz & Oliveira, 2004). It was observed that the anthers of these plants are empty or almost and they rarely produce fruits. Subterranean system is present in these populations. The inflorescence is branched as in *S.*

paniculatum, but the aculeous are acicular in the entire plant or only in its upper portion with sporadic curved and widened aculeous in some points. The calyx seems morphologically intermediate between those two species. Because of this morphological variation, the samples collected from these populations may be hybrids, and therefore shall be denominated in this study as "intermediate species".

Solanum variabile (known as *false* "*jurubeba*" and "*jurubeba-velame*"*)* is a perennial shrub and a very polymorphic species, erect and branched, armed on stems and branches with aculeous widened at the base, or nearly unarmed. The young leaves are ferruginous, and can be narrow or wide-lobed. Branches and leaves carry stellate trichomes with a typical pluricellular and pluriseriate pedicel which does not occur in the remaining species here analysed. Calyx with oval-shaped lobes, pointed at the apex. Corolla is white and shallowly lobed. Fruits erect. Probably it reproduces by seeds. This species is found in Paraguay and in Brazil, where it grows from the states of Minas Gerais and São Paulo to Rio Grande do Sul.

Solanum adspersum (common name not available) is a perennial shrub species, erect and few branched, armed on stems and branches with straight acicular aculeous and with slightly base widened aculeous. Its leaves are very similar to those from *S. guaraniticum*. Both species differ on the corolla lobes and on the position of its fruits. Corolla white, divided bellow the middle. Fruits pendant. *Solanum adspersum* is endemic and is only found in the Brazilian coastal regions of the states of São Paulo and Paraná, in the so called "restinga" vegetation.

Solanum tabacifolium (with the common names "*cardo-branco*", "*jurubeba"* and "*juveva*"), miscited as *S. asperolanatum* by Smith & Downs (1966), is a small perennial tree. Young stems and branches carry very small aculeous, slightly widened at the base.

Leaves concolour, abaxially covered by pubescence with two sizes of stellate hairs. Calyx covered by ferruginous trichomes, lobes wide with a narrow tip. Corolla is white, lobes deeply divided and two times longer then the calyx, also covered by ferruginous trichomes. Fruits erect. The reproduction is made by seeds. This species grows in Brazil in the Center Region (Mato Grosso and Goiás), South East Region (Minas Gerais, Rio de Janeiro and São Paulo) and in the South Region (Paraná and Santa Catarina).

Solanum bonariense L. (known as "*granadillo*" and "*naranjillo*" in Argentina) is a perennial shrub morphologically close to *S. guaraniticum*, from which it differs mainly in the indumentum and in the calyx morphology. *Solanum bonariense* has leaf trichomes sessile and porrect- stellate. The calyx lobes are narrow and long and when the corolla fall, the calyx lobes interweave. This species is found in Argentina and Uruguay and also in the state of Paraná, Brazil.

The aim of this study was to clarify the phylogenetic relationships and to measure inter taxonomic variability among some taxa of *Solanum* section *Torva.* To achieve these goals three types of markers were used: the nuclear and plastid markers (cpDNA) of which we used three genomic regions; the ITS spacers (internal transcribed spacer of the rDNA) of the nuclear genome (Desfeux & Lejeune, 1996); the *trn*L intron plus the *trn*L-*trn*F spacer (Taberlet *et al.*, 1991) and *trn*S-*trn*G (Hamilton, 1999) spacer of the cpDNA. The third marker corresponds to ISSR (Inter Simple Sequence Repeat) which was first described by Zietkiewicz *et al*. (1994) and is now widely used for genetic diversity studies (Davierwala *et al*., 2000; Panda *et al*., 2003), and even for phylogenetic analysis (Yockteng *et al.*, 2003). ISSR-PCR permits the detection of polymorphisms in the *locus* situated between the microsatellites using simple repeated sequences (SSRs) as primers (Wu *et al*., 1994; Zietkiewicz *et al*., 1994).

Materials and Methods

1) Material

Forty-two individuals were used for the phylogenetic analysis, being ten samples used as outgroup, the latter are classified in other sections of subgenus *Leptostemonum* (samples 33-41, Table 1). The distinct data sets were treated with a different number of taxa due to different reasons including the lack data and convention (Table 2). In analysis of nuclear and plastid markers were used three matrixes: **ITS matrix**- the ITS matrix was analyzed with 34 samples. The excluded sequences were: Section *Torva*: one sample of intermediate morphology species, four of *S. guaraniticum*, two of *S. paniculatum* and one of *S. variabile*. **Plastid matrix**- this matrix was analysed with 34 samples. The excluded sequences were: Section *Torva*: one sample of intermediate morphology species, three of *S. guaraniticum*, one of *S. paniculatum*, one of *S. variabile*; and outgroup: one of *S. aculeatissimum*, and one of *S. sisymbriifolium*. **Combined matrix**- 29 samples were analyzed in the matrix combining the three fragments. The excluded sequences were: Section *Torva*: two samples of intermediate morphology species, six of *S. guaraniticum*, two of *S. paniculatum*, one of *S. variabile*; and outgroup: one of *S. aculeatissimum* and one of *S. sisymbriifolium*.

Twenty-two samples were used to ISSR markers (Table 1), being used the species *S. viarum*, *S. atropurpureum* and *S. aculeatissimum* as outgroup.

2) DNA Isolation and amplification

The extraction of total DNA was realized with dry leaves in silica gel using the CTAB technique (Doyle & Doyle, 1990) suitably modified.

2.1) Nuclear and Plastid markers

For the phylogenetic analysis of the *Solanum* section *Torva* four fragments were used, of which one fragment corresponded to the ITS1 regions, the gene 5,8S and ITS2, and three fragments of plastid DNA: the intron of the gene *trn*L and the intergenic spacers *trn*L-*trn*F plus *trn*S-*trn*G.

For amplification of the fragments corresponding to the ITS region we used the "92" and "75" primers as described by Desfeux & Lejeune (1996); with PCR conditions as follows 1µl (30-100 ng) of DNA, 2.5 µl of reaction buffer 10x, 0.75 µl of $MgCl_2$ (50mM), 0.5 µl of dNTP (10mM), 0.25 µl of *Taq* DNA polymerase (5U/µl), 0.5µl of each primer (92 and 75), 2.5µl of DMSO (96%) and H_2O to make up 25 µl. The amplifications of this fragment were performed in the Applied Biosystems thermocycler (Gene Amp PCR System 2400) using the "Hot Start" PCR Method: 94°C for 5min, 72°C for 6 min; 35 cycles of 94°C, 45 sec., 58°C for 1 min, 72°C for 1 min and 30 sec; finishing with an extension at 72°C for 10 min. The PCR products were sequenced with the primers 92 and ITS3 (Desfeux & Lejeune, 1996).

The amplification of *trn*S-*trn*G region was realized with the primers "S" and "G" described by Hamilton (1999) under the following conditions: 2.5 µl of reaction buffer 10x, with 0.5 µl of dNTPs (10mM), 0.8 µl of $MgCl_2$ (50mM), 0.5 µl of each primer (10 pmol/µl), 0.2 µl of *Taq* DNA polymerase (5U/µl), 1 µl of DNA (30-50 ng) and H_2O to

make up a total volume of 25 µl. The conditions under which the amplification of this fragment was performed included an initial denaturing temperature of 94°C for 5 min; 40 cycles at 94°C for 1 min, 49°C for 1 min, 72°C for 1 min; ending with an extension at 72°C for 10 min. The PCR product was sequenced using the same primers as were used in the amplification.

The other two fragments, (intron of *trn*L gene and *trn*L-*trn*F spacer) were co-amplified and sequenced, using the primers "c" and "f" described by Taberlet *et al.* (1991) under the following conditions: 2.5 µl reaction buffer 10x, 0.5 µl of dNTPs (10mM), 0.75µl of $MgCl_2$ (50mM), 0.5 µl of each primer (10 pmol/µl), 0.25 µl of *Taq* DNA polymerase (5U/µl), 1 µl of DNA (30-50 ng), 1 µl of DMSO (96%) and H_2O to make up a total volume 25 µl. The amplification was realized with: initial denaturing of 94°C for 3 min; 35 cycles at 94°C for 1 min, 55°C for 1min., 72°C for 2 min; finishing with an extension at 72°C for 3 min.

2.2) ISSR Markers

To perform this study, four ISSR primers were used (Table 3) to infer the degree of variation between the different species belonging to the *Solanum* section *Torva.* The PCR reactions were carried out under the following conditions: 5µl of DNA (30-50ng), 2.5 µl reaction buffer 10x, with 1µl of dNTPs (10mM), 2.3 µl of $MgCl_2$ (50mM), 1 µl primer(100 pmol/µl), 0.2 µl of *Taq* DNA polymerase (5U/µl), 1 µl of DMSO (96%) and H_2O to complete a volume of 25 µl. The amplifications were performed in the thermocycler cited above with the following amplification programs: initial denaturing at 94°C for 5 min; 40 cycles at 94°C for 1 min, 50°C or 48°C – varying according to the primer involved- (Table

3) for 45sec, 72°C for 2 min; ending with an extension at 72°C for 5 min. As amplification products of the ISSR type segregate as dominant mendelian markers, the polymorphism was proven by the presence and absence of bands after the amplification and electrophoresis in a 1.8% agarose gel, stained with ethidium bromide and visualized and photographed under UV light. The size of the fragments was estimated by comparison with the Ladder 100bp (PB-L Produtos Bio-Lógicos).

3) Data analyses

3.1) Nuclear and Plastid markers

For the phylogenetic analysis of the nuclear and plastid markers we used four phylogenetic methods: Maximum Parsimony (MP), Maximum Likelihood (ML), Distance Analysis (D) were realized in PAUP 4.0b10 (Swofford, 2002), and Bayesian Inference (IB) using Mr.Bayes 3.0b4 program for Windows (Huelsenbeck & Ronquist, 2001). Three data sets were used: 1) ITS matrix (ITS1, 5.8S, ITS2); 2) Plastid matrix (intron of the *trn*L gene, *trn*L-*trn*F and *trn*S-*trn*G) intergenic spacers; 3) Combined Matrix (ITS matrix + plastid matrix). For all the matrixes MP, D, IB, and ML analysis were performed.

In addition, it was performed a parcimony analysis (gaps as fifth base) using only the sequence corresponding to the *trn*S-*trn*G spacer. In this analysis 38 samples were used adding sequences from other species from *Torva*: as *S. crinitipes* Dunal (AY998401), *S. glutinosum* Dunal (AY998418) and *S. lanceolatum* Cav. (AY998431).

Parsimony analyses were conducted using heuristic searches with tree-bisection-reconnection (TBR) branch swapping. Indels (insertion and deletion events) were treated either as missing data or as fifth character state. The Consistency (CI), Retention (RI),

Rescaled (RC) and Homoplasy (HI) indices were also calculated. The strength of support for individual tree branches was estimated using bootstrap values (BS) (Felsenstein, 1985) and decay indices (Bremer partition support method - PBS). Bootstrap values were from 1000 full heuristic bootstrap replicates. The Bremer partition support method was used to estimate how much each data set (nuclear and plastid regions) contributed to the formation of each branch in the total phylogenetic evidence in the combined matrix. The PBS values were calculated in PAUP and TreeRot.v2 programs (Sorenson, 1999).

To estimate the information contained for each data set, the g1 statistical test (Hillis, 1991) was realized from 1000 randomly trees generated by PAUP. The GC% content was also calculated in the same software. To test for congruence between two data partition (plastid and nuclear partitions), 1000 replicates of the partition homogeneity (PHT) test (essentially the incongruence length difference (ILD) test of Farris *et al.*, 1994, 1995), as contained in PAUP 4.0b10, were applied.

The appropriate models of nucleotide substitutions to perform ML and IB analyses were determined in ModelTest 3.06 program (Posada & Crandall, 1998) and the Akaike Information criterion (AIC). The models GTR+I+G, for ITS matrix, TVM+I+G, for Plastid Matrix, and GTR+I+G, for Combined Matrix were selected. The Bayesian analysis was conducted for 1,000,000 generations using the Markov Chain Monte Carlo method (MCMC). The ML analysis was estimated by a heuristic search with the *as-is* option.

Distance Analysis was conducted using the neighbour-joining (NJ) method (Saitou & Nei, 1987), with the model proposed by the manufacturer, ModelTest. This analysis was also realized in the same way in PAUP.

3.2) ISSR Markers

ISSR approach generated different fragment sizes that were treated as presence (1) or absence (0). Bands of identical size were assumed to be homologous throughout the species. The 0/1 binary matrix was used to calculate the similarity matrix using Dice and Jaccard coefficients. The phenogram was constructed with UPGMA using NTSYS 2.10 program (Rohlf & Marcus, 1993).

The Distance Analysis was inferred using the NJ algorithm and it was realized in PAUP.

Results

Nuclear data sets - ITS

ITS sequences for 34 samples showed an aligned length of 699 (missing data) and 691 (fifth data). The ITS matrix using gaps as fifth base showed a high number of PI = 201 (Table 2). All analyses based on ITS matrix *Solanum* section *Torva* appeared as monophyletic. In these analyses all samples of the intermediate morphology species are closely related to *S. guaraniticum* and *S. bonariense* (BS 74 for D analysis, results not showed). However, in the trees based on cloroplast and combined data, the intermediate morphology species is preferentially close to *S. paniculatum* (Figures 1 and 2).

ITS matrix presented a low index of homoplasy here, being phylogenetically informative. Among the different data sets analysed in the present work, the ITS matrix presented the lowest number of PI, and this is probably due to the length of this fragment which corresponds to a small region (in bases pairs, bp).

Chloroplast data

The combined sequences from intron *trn*L + *trn*L-*trn*F + *trn*S-*trn*G spacers (plastid genome) showed an aligned length of 1959 bp (gaps treated as missing data) and 1920 bp (gaps treated as fifth base). The plastid matrix corresponds to the highest PI= 364 (Table 2) when gaps were treated as fifth base.

Figure 1 showed the results obtained from IB analysis based on plastid data. In this figure as well as in other trees obtained from chloroplast data is observed that intermediate morphology species is close to *S. paniculatum* and *S. guaraniticum*, which may be its ancestors. This is also observed in figure 2 (MP form combined data).

Figure 1 showed also the group formed by *S. variabile* and *S. adspersum* that is also observed in other analyses based on chloroplast data (results not showed), however this group is not observed on the other analyses. *S. tabacifolium* does not form any stable group in analyses based on chloroplast data, appearing sometimes as the basal species of the section *Torva* with *S. torvum* (Figure 1).

In this analysis based on cpDNA data the section *Torva* appears as monophyletic supported by BS 91 (Figure 1).

The monophyly is also maintained with a more complete sampling when *S. crinitipes*, *S. glutinosum* and *S. lanceolatum* where added and a MP analyses was performed based on the *trn*S-*trn*G spacer. This analysis showed an aligned length of 730 characters, with 32 characters being parsimony-informative (PI) and the most parcimonious trees were produced with 276 steps. This analysis showed a high consistency index (CI = 0.74), and divided the section *Torva* into three groups: the first one is formed by *S. paniculatum*, intermediate morphology species, *S. adspersum* and *S. variabile* (supported by BS 92); the second is formed by *S. guaraniticum*, intermediate morphology species, *S. bonariense*, *S. tabacifolium* and *S. torvum* (BS 51); and the third group is formed by *S.*

crinitipes, *S. glutinosum* and *S. lanceolatum* (BS 90). The two first groups are closely related and this groupment is supported by BS 81, being the other three species a sister group of the remainder species from section *Torva* (BS 88).

All data sets combined (total evidence)

The most phylogenetic information was provided by MP analysis based on the combined matrix, this analysis supplied the greater number of PI and the g1 value obtained by the Hillis test was -0.83.

Figure 2 showed the majority consensus tree from MP obtained from the total evidence. The combined matrix showed an aligned length of 2,652 bp (using missing data) and 2,611 bp (gaps as fifth base). The MP analysis performed from this latter matrix obtained the highest PI = 552 (Table 2).

All analyses based on the total evidence showed that *Solanum* section *Torva* was monophyletic (BS 100, PBS = 11, Figure 2). In all performed analyses a group formed by *S. guaraniticum*, *S. bonariense* and one individual from the intermediate species was maintained, as in cpDNA and ITS matrixes. The majority consensus tree of all MPT (most parcimonious trees) (Figure 2) showed that plastid DNA contribute more to its formation than the ITS region supported by PBS.

Differences on the topology of the trees obtained by MP analyses were observed when the different indels treatments were considered. Figure 2 showed a clade grouping *S. paniculatum* and *S. paniculatum* x *S. guaraniticum* (BS 100) using gaps as fifth base or missing data. Nevertheless, when indels are treated as fifth base (Figure 2), *S. tabacifolium* is more close to this group (supported only by BS 78).

The position of *S. variabile* is very affected by the indels treatment, *S. variabile* forms a separate group when indels are treated as fifth base (BS100, Figure 2). However, this species is grouped to *S. adspersum* and *S. tabacifolium* when gaps are treated as missing data.

ML and IB analyses showed similar topology (results not showed). The trees differed from MP (Figure 2) by the position of *S. variabile* and *S. paniculatum* that are closely related in these analyses and this group is also evidenced by ITS. Other difference concern the group formed by *S. adspersum* and *S. tabacifolium.* In these trees *S. paniculatum* x *S. guaraniticum* appears as an isolated group and closed to *S. guaraniticum.*

Genetic variability intra and inter-specific in *Solanum* Section *Torva*

The ITS matrix was also used to verify the genetic variability of the section *Torva*. Variability intra- and inter-specific were analysed when two or more ITS sequences were used. The intermediate morphology species showed the great number of variation intra-specific with 110 variable characters, followed by *S. variabile* with 80, *S. guaraniticum* with 57, *S. adspersum* with 42 and *S. paniculatum* with 21 variable characters. The size of ITS sequences varied from 600 to 640 bp within this species. It showed a high intra-specific variation within this taxon, once it should present the same sequence among all analysed individuals. This variation may be mainly due to different local origins and to distinct selection pressures by individuals of a same species. ITS matrix corresponding to the section *Torva* showed an alignment of 658 bp, being 377 bp variable, and 313 bp parsimony informative sites. A high variation inter-specific was showed among the eight

species (circa of 54 to 57% of variation among ITS sequences) from section *Torva* (including intermediate morphology species).

All primers used to perform ISSR analyses allowed DNA amplification with a clear identification of the bands and provided information about the five species of S*olanum* section *Torva* used in this study. The present work can be used as a reference to other works concerning genetic variability in *Solanum*. The primers generated a total of 82 fragments (bands) for 22 individuals used in this study (Table 1). The fragments varied in size from 250 bp to more than 1kbp (Table 2). Of the 82 ISSR bands obtained, only one was present in all individuals and the remainder (99%) were polymorphic. Figure 3 showed an amplification pattern obtained to the primer 4 for 22 analysed samples.

The minimum and maximum number of fragments generated per primer was 18 (primers 3 and 4) and 27 (primer 2), respectively (Table 4), with an average of 20.5 fragments. Considering all analyzed primers (Table 4) among the species, *S. adspersum* presented the highest number of bands (40 bands), while *S. aculeatissimum* the lowest (17 bands). Twenty-five marker bands (MB) were obtained for 22 samples, of which 21 MB were exclusively of the section *Torva.* The species presenting the highest number of marker bands were *S. adspersum* (with ten MB, being two diagnostic bands), *S. guaraniticum* (four MB) and *S. paniculatum* x *S. guaraniticum* (three MB) (Table 4).

Figure 4 showed a dendogram calculated from similarity coefficients of Dice. The dendogram illustrates genetic similarities among species from section *Torva*, in which *S. adspersum* corresponds to the more distant species from other taxa. There is a large group in the dendogram including almost all species from the section *Torva* collected in the brazilian state of Rio Grande do Sul, and exclude species from *Torva* collected in other geographic regions plus a sample of *S. guaranitum* which has a distinct morphology and a

sample of the intermediate species. This group lies *S. paniculatum* and the intermediate morphology species and reveals an intra-specific variation of *S. guaraniticum*, which appears dispersed in the dendogram, coming closer both to *S. paniculatum* and to *S. variabile*. The species from section *Acanthophora* showed more close to the species from section *Torva* than to *S. adspersum* (collected in the brazilian state of Paraná).

The separation of *S. adspersum* of other species from *Torva* can be seen in the analysis of the principal coordinates (Figure 5), and in the dendogram (Figure 4). This is probably due to the high number of exclusive bands (markers) of this species obtained by ISSR. In addition, it can be observed that the interaction between the intermediate morphology species and their supposed progenitors *S. paniculatum* and *S. guaraniticum* is seen in morphological and molecular evidences.

In NJ analysis the section *Torva* also appears as monophyletic (Figure 6). The corresponding dendogram presents two large groups: the first one is formed by *S. paniculatum*, *S. variabile*, *S. guaraniticum* and the intermediate morphology species (*S. paniculatum* x *S. guaraniticum*), all of them are typical representatives of *Solanum* section *Torva* found in the brazilian state of Rio Grande do Sul; and the second group is formed by *S. bonariense* (Argentina), *S. tabacifolium* (Minas Gerais) and *S. adspersum* (Paraná), plus one individual of *S. guaraniticum* that is almost without aculeous in the base (Rio Grande do Sul, Brazil) - showing an inter-specific variability within *Torva*.

Discussion

Nuclear and Plastid Markers

Solanum section *Torva* appears as monophyletic in almost all analyses performed in this study and it is in agreement with Levin *et al.* (2006). This monophyly is maintaining even when a more complete sampling from section *Torva* is analysed.

The most frequent relationships among the species from section *Torva* were as follow: (i) the group concerning *S. guaraniticum* + *S. bonariense* + a sample of intermediate morphology – this occurred in 93% of the trees; (ii) *S. adspersum* + *S. tabacifolium* (60%); (iii) *S. paniculatum* + the intermediate morphology species (46%); and (iv) *S. paniculatum* + *S. variabile* (40%).

Solanum guaraniticum, *S. bonariense* and "*S. paniculatum* x *S. guaraniticum"* are very close, which appeared as an isolated group in almost all analyses. The clade concerning "*S. paniculatum* x *S. guaraniticum"* plus *S. guaraniticum* can be explained by the supposition that this latter species is considered one of the possible progenitors of the species with intermediate morphology. The morphological traits of "*S. paniculatum* x *S. guaraniticum*" is intermediate between *S. paniculatum* and *S. guaraniticum* including aciculate aculeous even on the apex branches (as in *S. guaraniticum*), and a calyx with a intermediate form between those of *S. paniculatum* and *S. guaraniticum*

The strong relationship between *S. bonariense* and *S. guaraniticum* can be explained in part by some morphological similarities shown by these species and by six synapomorphies showed by molecular data. PBS analysis performed from MP tree based on total evidence showed that the plastid markers (which are in general more conservative than ITS) contributed more to the formation of this group.

The species collected in the brazilian states of Paraná (*S. adspersum)* and Minas Gerais (*S. tabacifolium*) are grouped in almost all phylogenetic analyses based on both ITS

and combined matrixes. However, the analyses based on the plastid matrix this relationship is not confirmed.

The third most frequent interaction among the species from section *Torva* is that between *S. paniculatum* and the intermediate morphology species. The latter shares morphological characters with *S. paniculatum*, which is supposed be one of its progenitors. This interaction, however, is only observed in the analyses from chloroplast and combined matrixes. The relationship between the intermediate morphology species and its supposed progenitors differ according to the matrix used for the phylogenetic analyses – it is probably due to different types of inheritance of each marker. The plastid genome generally is inherited as uniparental, being maternal in Angiosperms, while the nuclear genome has biparental inheritance. However, there are some reports where the plastid DNA can be inherited by biparental inheritance (Brent & David, 1989, Avni & Edelman, 1991, Shore & Triassi, 1998; Chen *et al*., 2002). In such cases, it is easier to understand the position of the intermediate morphology species with both supposed progenitor found in the analyses derived from the chloroplast matrix. This interaction is also observed and very well supported (BS 100) in the analysis of the combined matrix independently of the treatment of gaps.

In the fourth most frequent relationship *S. paniculatum* is related to *S. variabile*, it is mainly evidenced in the trees obtained from ITS data. ML and IB analyses from the combined matrix also showed this relationship.

All data matrixes used in this study equally contributed to the elucidation of the phylogenetic relationships among the species of *Solanum* section *Torv*a. PBS analyses showed that both matrixes (nuclear and plastidial) allowed the construction of the parsimony tree based on the combined matrix. In addition, another PBS test was performed

to quantify the contribution of each chloroplast fragment in the formation of the clades. This test showed that the information of the fragment *trn*L-*trn*F plus *trn*L intron was more than twice that provided by *trn*S-*trn*G spacer. This data confirms MP analyses from each region, in which *trn*L intron + *trn*L-*trn*F spacer showed a high PI in comparison with *trn*S-*trn*G intergenic spacer. This is probably due to the size of these plastidial sequences because the CI in both regions was high independently of how the indels were treated.

All performed analyses conducted from the same matrix showed near the same topology besides some divergences were observed. Concerning the probabilistic analyses (ML and IB), the corresponding trees presented similar topologies independent of the matrix used to infer the phylogeny. So, we can conclude that is not necessary to perform both analyses, mostly when the sampling is very large and the ML is very time consuming. Nowadays the number of phylogenetic researchers giving preference to Bayesian Inference over Maximum Likelihood has increased. This is mainly due to the rapidity of the method (IB) in comparison to ML and the former permits estimate the model of evolution using the same technique. The present work bears out the importance in use more than one method of phylogenetic reconstruction. The trees generated by different methods (Probabilistic, Parsimony and of Distance), using a combined matrix, present considerable statistical support, although some topology differences can be observed.

Congruency among the markers (nuclear and plastid)

The Partition Homogeneity Test (PHT) disclosed incongruence among nuclear and plastidial markers. (P = 0.001). This is probably due to differences in size of the two partitions (chloroplast = 1848 bp; ITS region = 691bp). However, with the PBS analysis both regions are congruent. This congruency may be observed by the PBS values in which

only two divergences are observed in the 27 clades generated (results not showed). The congruency between these two marker types, in this analysis, is probably due to the high indexes of CI and PI in both matrixes, to the similar values obtained with the Hillis test, and to the homogeneity presented by the sequences representing the species from section *Torva.*

Genetic diversity intra and inter-specific in *Solanum* Section *Torva*

The high genetic variation showed among the species from section *Torva* by both ITS and ISSR markers must be due the following reasons: This reasons concern the distinct morphology showed within one same species, different places of collection, and to different floral biology and sexual shape presented within section *Torva*. Therefore, although many species of *Solanum* has monoclinic typical flowers, some variation is noted in sexual shape, including andromonoecy, androdioecy and dioecy (Anderson 1979, Coleman & Coleman 1982, Oliveira Filho & Oliveira 1988, Anderson & Symon 1989). Within section *Torva* were described already andromonoecious and monoecious species (Symon, 1979). This difference in floral and reproductive shapes allowing the supposed high variation to the section *Torva* mainly referring *S. paniculatum* as allogamous species (Forni-Martins *et al*., 1998). The allogamous species possess a system of gametophytic incompatibility (Pandey 1960, Heslop-Harrison 1975). Within section *Torva*, the genetic incompatibility is known only to *S. paniculatum*, while *S. hispidum* (Baksh & Iqbal 1978) and *S. torvum* (Hossain 1973) are auto-compatible. This difference among the species may lead to reproductive isolations and/or geographic isolation as showed by species from section *Torva* in this work.

The ITS region showed be a good marker to verify the genetic variation intra and inter-specific within the section *Torva*. The high variation within the intermediate

morphology species is mainly due to distinct morphologies observed within this taxon being sometimes similar to *S. guaraniticum* or to *S. paniculatum.* Moreover, this variation within species from the section must be to different places of collection and to concerted evolution present by this marker, homogenising the DNA sequence to one supposed parental (Chase *et al.*, 2003).

ISSR markers showed a high level of polymorphism for the species from *Solanum* section *Torva.* Moreover, its also provided evidence on the considerable inter-taxonomic variability of the studied species, as can be seen in the dendogram obtained by similarity. Furthermore, we also confirmed a high degree of intra-taxonomic variation for almost all species. This intra-taxonomic variation was observed most clearly within *S. guaraniticum*, in the five samples analyzed. This species is found dispersed throughout the dendogram interacting with various species from *Solanum* section *Torva*. This dispersion may be explained by the distinct morphological variations shown by this species mainly regarding the presence of aculeous. One of the observed *S. guaraniticum* relationships was with *S. bonariense.*

Solanum bonariense (Argentine and Uruguay) and *S. guaraniticum* (southern Brazil) contain toxic substances which cause neurological disturbances in cattle (Riet-Correa, 1982; Riet-Correa *et al*., 1983). The name *S. bonariense* was always well defined and no doubts were ever raised about the identification of this species. However, *S. guaraniticum* was generally cited in the literature from southern Brazil under the name of *S. fastigiatum* Willd. Two varieties of *S. fastigiatum* were referenced (Smith & Downs, 1966). The typical variety, *S. fastigiatum* var. *fastigiatum* was characterized by the presence of few aculeous, while *S. fastigiatum* var. *aciculare* has numerous aculeous over all the plant. Because the work of Smith & Downs (1966) is widely used in southern Brazil these names

are generally accepted. However, according to Mentz & Oliveira (2004), *S. fastigiatum* var. *fastigiatum* corresponds to *S. bonariense*, while *S. fastigiatum* var. *aciculare* corresponds to *S. guaraniticum*. On the other hand, the present study showed that *S. guaraniticum* is close related to *S. bonariense* bases on the analyses of similarity and phylogeny. This is probably due to the high degree of similarity concerning the morphology of these taxa.

In addition to the inter and intra-taxonomic variation shown by ISSR markers, the analysis of similarity separated *S. adspersum* (collected in the brazilian state of Paraná) from the large group formed by the species from section *Torva* (all of them collected in the brazilian state of Rio Grande do Sul), and placed *S. adspersum* as an outgroup. This position is partially due to the large number of exclusive bands generated for this species in comparison to other taxa, and to different evolutionary processes brought about the geographical separation.

The great contribution to a better understanding of the species from section *Torva* provided by ISSR markers may serve as a starting point for future studies using this marker to determine the taxonomic variation among other species from section *Torva*, including intra-taxonomic level.

Conclusion

Solanum section *Torva* has been shown monophyletic, at least as far as the species utilized in this study are concerned which provide an evolutionary history of this section from southern region of Brazil. We found four most-frequent groups among the species from section *Torva* - all of them were with a strong support in all performed analyses. The knowledge of these relationships will contribute to understand the biology of these species and assist possible programs of conservation and maintenance of the local biodiversity.

ISSR markers were found especially informative and polymorphic among the studied species from section *Torva*. We believe that this work will serve as a basis for future investigations about *Solanum* section *Torva*. The nuclear and plastidial markers help us to the elucidation of taxonomic problems and for the verification of genetic diversity among the studied taxa. This work stands, therefore, as a starting point to understand the evolutionary history of the species from *Solanum* section *Torva*, contributing to their recognition as important components of the Brazilian native plants.

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Table 1: List of species from *Solanum* section *Torva* used in phylogenetic analysis and ISSR analyses. The asterisks indicate fragments sequenced (or presence of data in ISSR) for each sample and N to the lacked data. The samples of number 33-42 in the table comprise the species of other sections from *Solanum* subgenus *Leptostemonum*, which were used as outgroup.

	Samples	Voucher	ITS (GenBank accession numbers)	*trn*L-*trn*F plus intron *trn*L (GenBank accession numbers)	*trn*S-*trn*G (GenBank accession numbers)	ISSR Markers	Sampling Places
1	*Solanum paniculatum* X *Solanum guaraniticum*	L. A. Mentz, T.T Souza-Chies and R.B. Miz, 301.	*	*	*	*	RS, Porto Alegre.
2	*Solanum paniculatum* X *Solanum guaraniticum*	L. A. Mentz, T. T. Souza-Chies and R. B. Miz, 302.	*	*	*	*	RS, Porto Alegre.
3	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 309 - ICN	*	*	*	*	RS, Passo Fundo, Fazenda Sementes and Cabanhas Butiá
4	*Solanum paniculatum* X *Solanum guaraniticum*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, 305-ICN	**N**	*	*	**N**	RS, Viamão, Parque Estadual of Itapuã
5	*Solanum paniculatum* X *S. guaraniticum.*	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S .Vendruscolo, R.B. Miz, 311 - ICN	*	*	**N**	**N**	Estrada Carazinho/Ihuí – near of Rio Colorado.
6	*Solanum guaraniticum* A. St.-Hil.	T. T. Souza-Chies, 221.	*	*	*	*	RS, Antônio Prado. Estrada Amarilho. Propriedade of Sônia Montanari
7	*Solanum guaraniticum* A. St.-Hil.	T.T. Souza-Chies, 222	*	*	**N**	*	RS, Monte Bonito, distrito of Pelotas. Propriedade Águas Claras.
8	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 344 – ICN.	*	*	*	*	RS, Venâncio Aires (65km)
9	*Solanum guaraniticum* A. St.-Hil. (almost without spines)	L.A. Mentz, E.L.C. Soares, R.B. Miz, 349.	*	*	*	*	RS, Santa Cruz
10	*Solanum guaraniticum* A. St.-Hil. (plant with spines in stem and leaves)	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B.	*	*	*	*	RS, Fontoura Xavier, BR 386, km 276

		Miz, 336- ICN					
11	*Solanum guaraniticum* A. St.-Hil. (plant with spines only in low part of stem)	L.A. Mentz, E.L.C. Soares, R.B. Miz, 345a.	*	*	*	**N**	RS, Santa Cruz
12	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 345b	*	*	**N**	**N**	RS, Santa Cruz
13	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 345c.	*	*	*	**N**	RS, Santa Cruz
14	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 339.	*	*	**N**	**N**	Coronel Barros - Parada Lajeado do Tigre –BR285 Km 476
15	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B. Miz, 333.	*	*	*	**N**	RS, Santo Ângelo
16	*Solanum guaraniticum* A. St.-Hil.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 348.	*	*	*	**N**	RS, Santa Cruz
17	*Solanum paniculatum* L.	R.B. Miz, 101.	*	*	*	*	RS, Porto Alegre
18	*Solanum paniculatum* L.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, 304-ICN.	**N**	*	*	*	RS, Viamão, Parque Estadual de Itapuã
19	*Solanum paniculatum* L.	M. F. Agra, K. Nurit and I. Basílio. 6311	*	*	**N**	*	Paraíba, Município João Pessoa, Campus da Universidade Federal of Paraíba
20	*Solanum paniculatum* L.	L.A. Mentz, E.L.C. Soares, R. B. Miz, 341- ICN.	*	*	*	**N**	RS, Triunfo
21	*Solanum paniculatum* L.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 340- ICN.	*	*	*	**N**	RS, Triunfo
22	*Solanum variabile* Mart.	L. Essi, 301.	*	*	*	*	RS/SC, Cambará do Sul. Serra do Faxinal.
23	*Solanum variabile* Mart.	L. Essi, 302	*	*	*	*	SC, Mauro Müller.
24	*Solanum variabile* Mart.	L. Essi, 304	*	*	*	*	SC, Bom Jardim da Serra. Serra do Rio do Rastro
25	*Solanum variabile* Mart.	L. Esii, 319	*	*	*	**N**	SC, Urubici
26	*Solanum variabile* Mart.	L. Essi, 320	*	*	**N**	**N**	SC, Urubici
27	*Solanum torvum* Sw.	_	* (AF244729)	* (AY266246)	* (AY555478)	**N**	GenBank
28	*Solanum bonariense* L.	G.E. Barboza, E.M. Fillipa, F. Chiarini, E. Marini, 1568.	*	*	*	*	Argentina Entre Rios: Department Gualeguaychú, Desde Ceibas rumbo a Villa Paranacito.
29	*S. tabacifolium* Dunal	I.M. Palhares, J.R.					MG, Belo Horizonte

		Stehmann. 94050	*	*	*	*	
30	*S. adspersum* Witasek (plant with fruit)	L.A. Mentz , J.R. Stehmann, 4185	*	*	*	*	Paraná, Potinga
31	*S. adspersum* Witasek (plant with flowers and fruits)	L.A. Mentz, J.R. Stehmann, 4186a.	*	*	*	*	Paraná, Potinga
32	*S. adspersum* Witasek (sterile plant)	L.A. Mentz, J.R. Stehmann, 4186b.	*	*	*	*	Paraná, Potinga
33	*S. atropurpureum* Schrank	L.A. Mentz, E.L.C. Soares, R. B. Miz, 343-ICN.	*	*	*	*	RS, Santa Cruz
34	*S. aculeatissimum* Jacq.	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R. B. Miz, 319- ICN.	*	*	*	*	RS, Passo Fundo. Fazenda Sementes e Cabanhas Butiá.
35	*S.viarum* Dunal	L.A. Mentz, E.L.C. Soares, R.B. Miz, 346- ICN.	*	*	*	*	RS, Santa Cruz
36	*S. vaillantii* Dunal	L.A. Mentz, E.L.C. Soares, M. Vignoli-Silva, G.S. Vendruscolo, R.B. Miz, 310- ICN.	*	*	*	**N**	RS, Passo Fundo, Fazenda Sementes and Cabanhas Butiá.
37	*S. sisymbriifolium* Lam.	L.A. Mentz, E.L.C. Soares, R.B. Miz, 351-ICN.	*	*	*	**N**	RS, Porto Alegre – Campus do Vale - UFRGS
38	*S. sisymbriifolium* Lam	_	* (AY561271)	* (AY266235)	* (AY555473)	**N**	GenBank
39	*S. melongena* L.	_	* (AF244726)	* (AY266240)	* (AY555465)	**N**	GenBank
40	*S. robustum* Wendl.	_	* (AY561270)	* (AY266259)	* (AY555472)	**N**	GenBank
41	*S. jamaicense* Mill.	_	* (AF244724)	* (AY266239)	* (AY555472)	**N**	GenBank
42	*Solanum stagnale* Moric.	_	* (AY561272)	* (AY266262)	* (AY555474)	**N**	GenBank

Table 2: Comparison among nuclear and plastid data from *Solanum*.

	ITS Matrix		Plastidial Matrix		Combined Matrix	
Number of individual	34		34		29	
Hillis test	g1 = - 0.77		g1 = - 0.73		g1 = - 0.89	
Content of GC (%)	0.70		0.33		0.44	
Índels treated with date	missing	fifth base	missing	fifth base	missing	fifth base
Aligned lenght including or excluded gaps	699	691	1959	1920	2652	2611
Tree lenght	381	607	417	959	773	1562
Number of characters constants	471	400	1661	1296	2155	1713
PI	126	201	131	364	246	552
CI	0.74	0.69	0.78	0.78	0.75	0.72
HI	0.25	0.31	0.21	0.22	0.24	0.27
RI	0.80	0.80	0.81	0.86	0.77	0.80
RC	0.60	0.55	0.64	0.67	0.58	0.58

PI = Number of parsimony – informative characters.

Table 3: ISSR primers, sequences, annealing temperature, number of fragments scored and approximate size range (in base pairs) of the fragments resulted from each primer in 22 individuals (Table 1).

Primers	Sequence	Annealing temperature (°C)	Number of fragments scored	Number of polymorphic fragments	Fragment size range (bp)
1	5'-CTC TCT CTC TCT CTC TG-3'	48	19	19	400pb- +1kb
2	5'- ACA CAC ACA CAC ACA CT- 3'	50	27	27	300pb- +1 Kb
3	5'- GAG AGA GAG AGA GAG AT-3'	50	18	18	350pb - +1 kb
4	5'- CTC CTC CTC CTC RC-3'	50	18	17	250pb - + 1kb
	TOTAL	-	82	81	-

Table 4: Number of fragments scored for each taxon to each indicated primer and respective markers bands (fragments) scored in parenthesis.

Taxa	Number of individuals	Primer 1	Primer 2	Primer 3	Primer 4	Total
S. paniculatum x *S. guaraniticum*	3	8 (0)	13 (3)	5 (0)	5 (0)	**31 (3)**
S. guaraniticum	5	9 (1)	12 (0)	7 (0)	10 (3)	**38 (4)**
S. paniculatum	3	9 (0)	8 (0)	4 (0)	5 (0)	**26 (0)**
S. variabile	3	8 (1)	8 (0)	7 (0)	6 (0)	**29 (1)**
S. tabacifolium	1	3 (0)	11 (1)	6 (0)	5 (0)	**25 (1)**
S. adspersum	3	11(4)	13 (3)	9 (1)	7 (2)	**40 (10)**
S. bonariense	1	3 (1)	8 (0)	7 (1)	3 (0)	**21 (2)**
S. viarum	1	4 (0)	14 (1)	3 (0)	8 (1)	**29(2)**
S. atropurpureum	1	3 (0)	4 (0)	5 (1)	9 (1)	**21 (2)**
S. aculeatissimum	1	3 (0)	5 (0)	3 (0)	6 (0)	**17 (0)**
Total	22	(7)	(8)	(3)	(7)	**(25)**

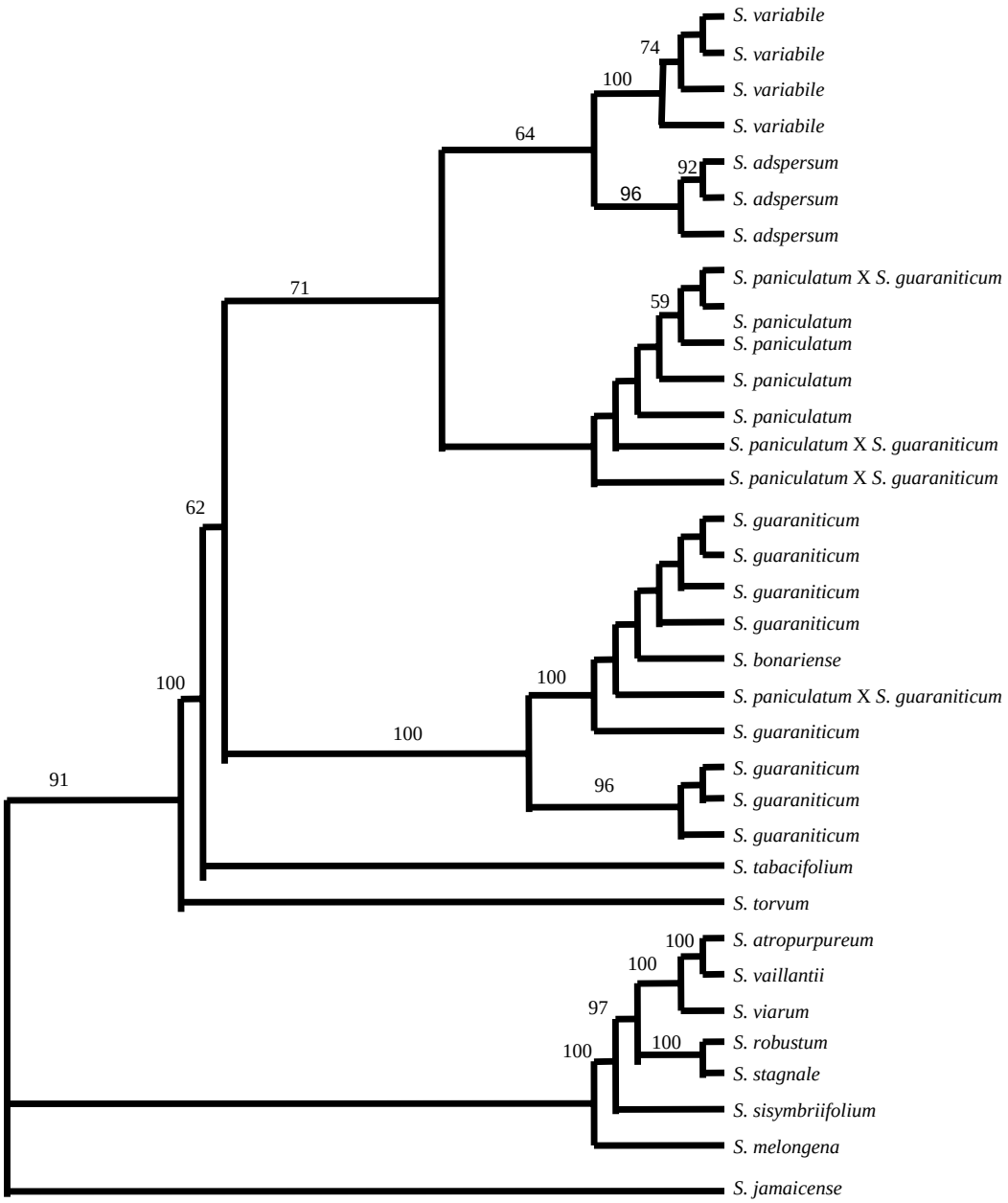

Figure 1: Majority consensus tree obtained from Bayesian analysis based on the plastidial matrix. The posterior probability for each clade is indicated above its respective internal branch (when higher than 50%).

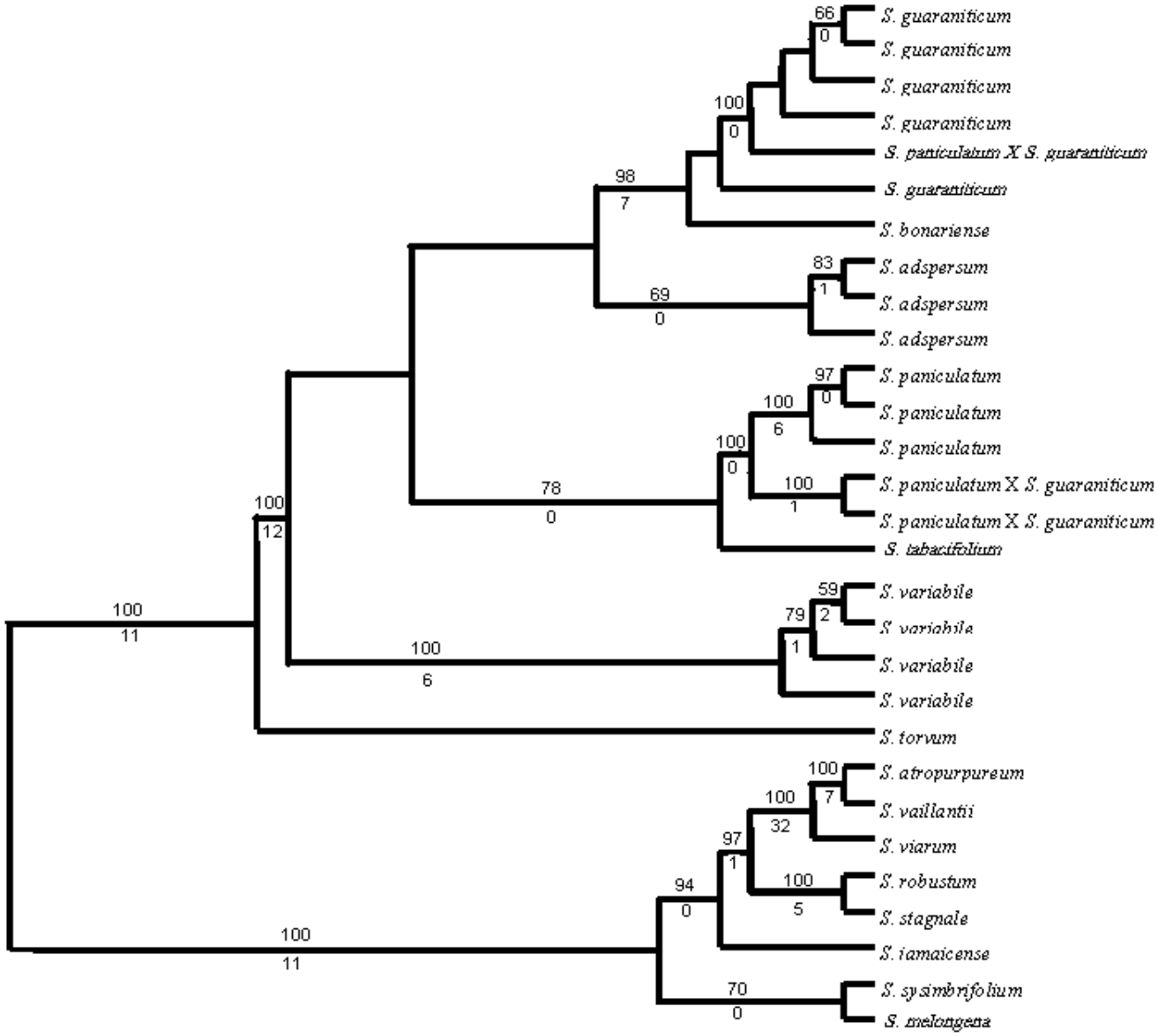

Figure 2: Majority consensus phylogenetic tree from maximum parsimony analysis (indels treated as fifth base) from the combined matrix. Bootstrap values >50% are shown above the branches, decay indices below.

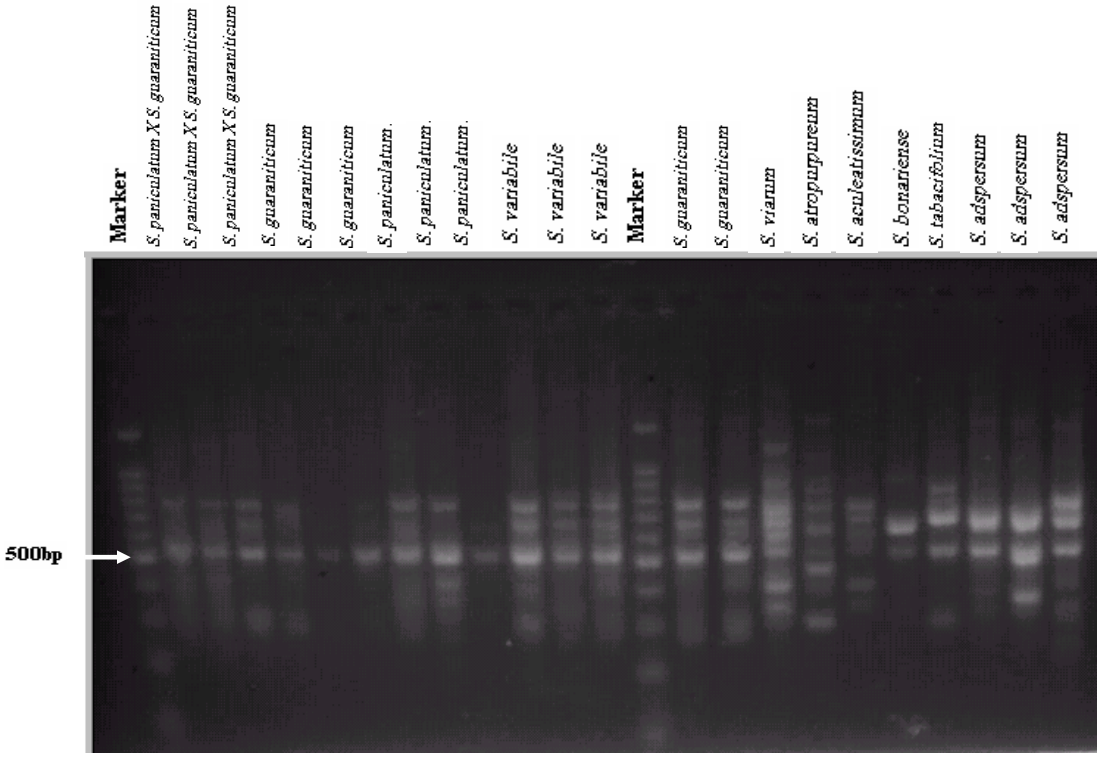

Figure 3: Inter-simple sequence repeat (ISSR) patterns visualized on agarose gel (1.8%) for 22 samples using the primer 4. Marker = molecular-size marker (100-base pairs ladder, PB-L Produtos Bio-Lógicos).

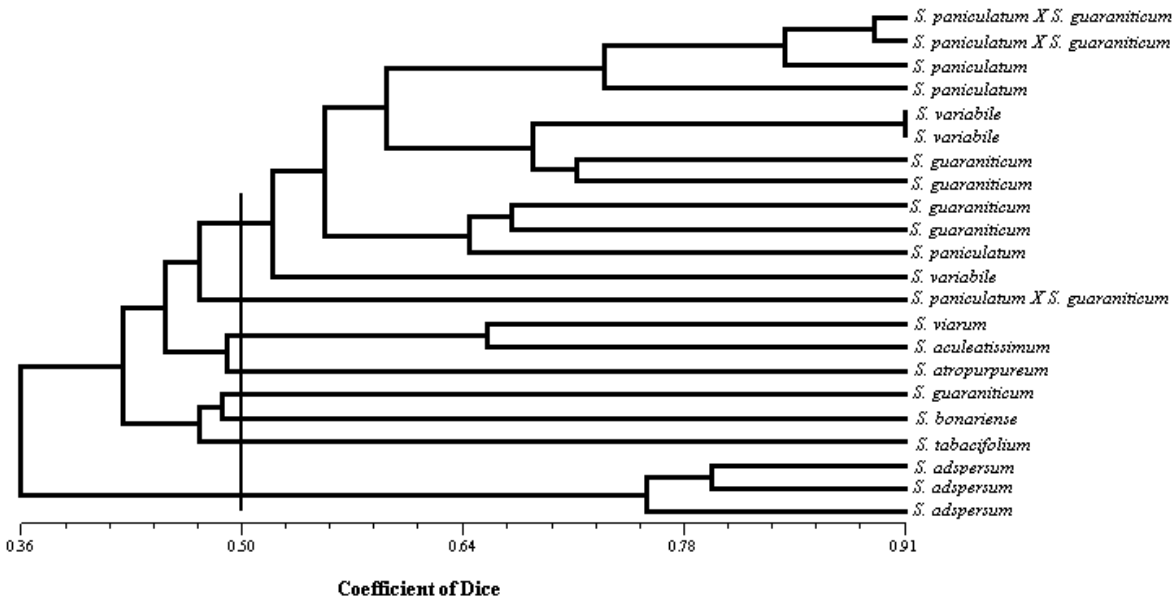

Figure 4: Dendogram based on the Coefficient of Dice showing genetic relationship among 22 individuals based on ISSR markers. The vertical bar corresponds to the mean similarity.

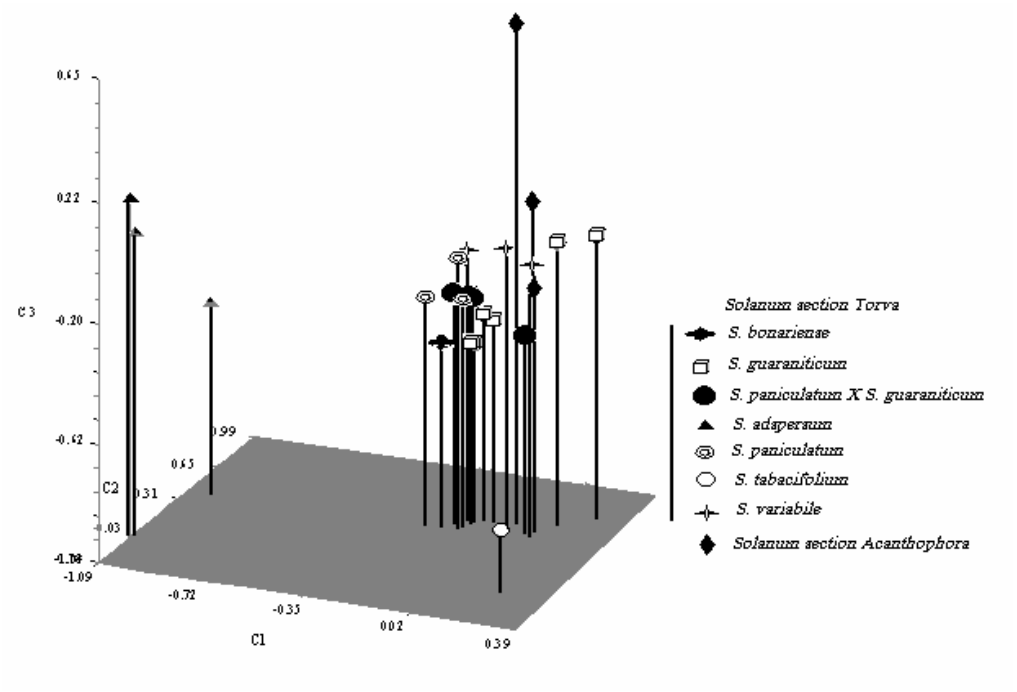

Figure 5: Principal Coordinates Analysis (PcoA) plot 3D based on ISSR data.

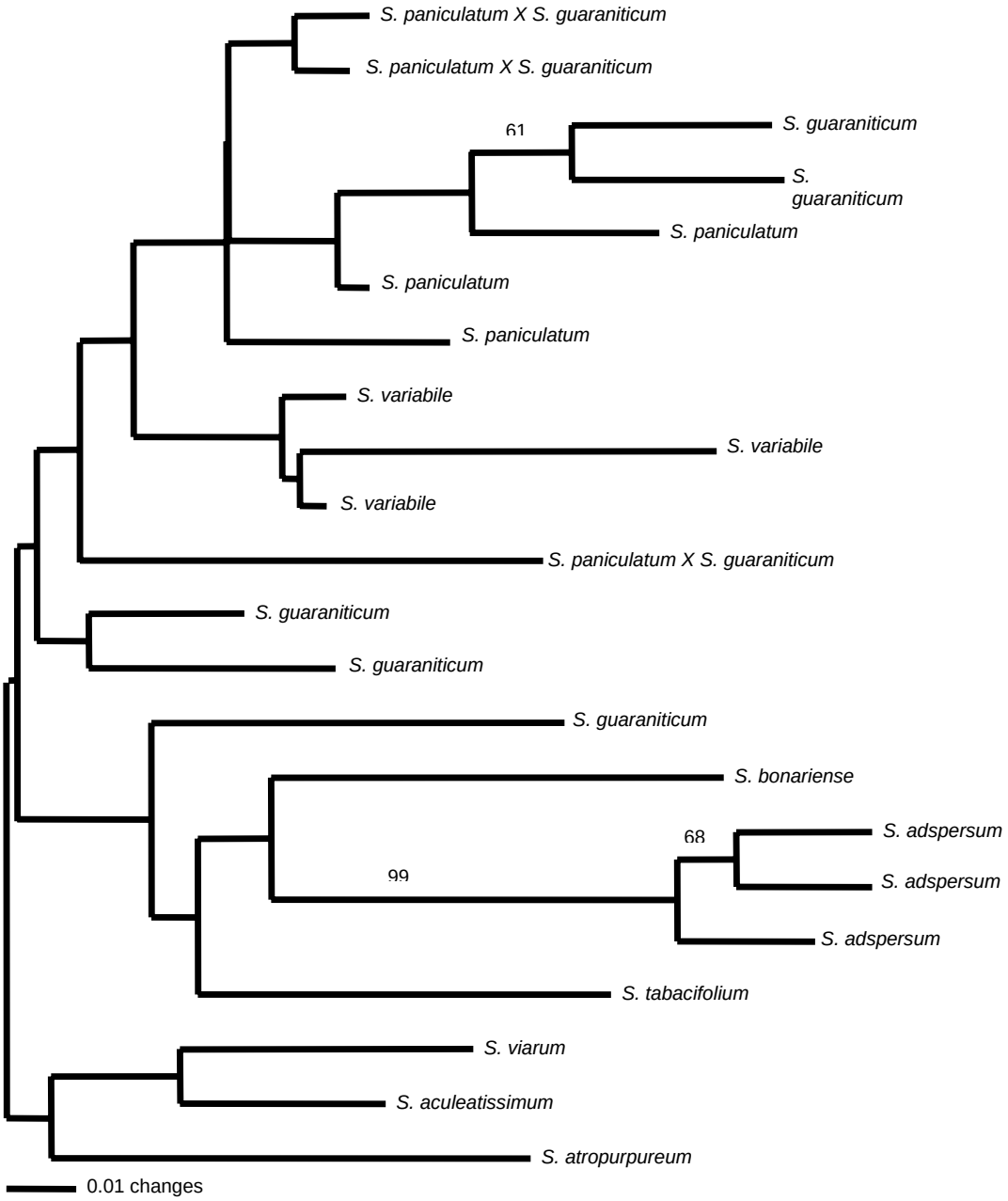

Figure 6: Neighbor-Joining tree obtained from ISSR Numbers above branches indicate bootstrap support values.

5.- Discussão

A execução do presente trabalho possibilitou a inferência das relações filogenéticas mais freqüentes entre as espécies da seção *Torva*, como comentado no artigo 2. Tais inferências são apresentadas nas árvores filogenéticas inseridas nos artigos 1 e 2, e grande parte delas é ilustrada nos anexos (capítulo 8) desta dissertação.

As diferentes análises realizadas com base em diferentes marcadores evidenciam a importância da utilização de mais de um tipo de marcador (preferencialmente de genomas distintos) e mais de um método de inferência filogenética. Foi observada na topologia das árvores uma constância na formação de grupos ao analisarmos filogenias obtidas com o mesmo tipo de marcador, A comparação de topologias oriundas de diferentes análises filogenéticas leva à obtenção de conclusões com maior probabilidade de serem as verdadeiras.

Atualmente, trabalhos que buscam o conhecimento sobre a filogenia de plantas têm utilizado informações oriundas de marcadores nucleares e plastidiais. O principal motivo desta utilização é a origem distinta de cada marcador, os quais têm proporcionado uma visão mais ampla da história evolutiva das espécies.

Dentro da seção *Torva* ficou clara a relação de parentesco entre os indivíduos que apresentam uma morfologia intermediária (*Solanum paniculatum* x *Solanum guaraniticum*) com seus supostos progenitores *S. paniculatum* e *S. guaraniticum.*. Porém, as relações variaram conforme o tipo de marcador utilizado nas análises. Nas análises filogenéticas realizadas com os marcadores plastidiais observou-se uma interação dos indivíduos morfologicamente intermediários com ambos os progenitores (artigos 1 e 2), esta relação também ficou evidenciada utilizando os marcadores ISSR (artigo 2). Entretanto, nas análises com ITS, apenas *S. guaraniticum* apresentou uma relação de parentesco com os indivíduos de morfologia intermediária. Tais diferenças resultam provavelmente do tipo de herança dos marcadores nucleares e plastidiais, e ao grau de homologia entre as seqüências. Pois, freqüentemente o genoma nuclear tem sido descrito como de

origem bi-parental e o genoma plastidial de origem uni-parental materna para as Angiospermas. Porém, em nosso trabalho, ambos os progenitores aparecem relacionados com os indivíduos de morfologia intermediária nas análises de cloroplasto, o que pode ser explicado por uma possível herança bi-parental do genoma plastidial. Essa característica bi-parental do genoma plastidial vem sendo detectada e testada em vários trabalhos. No traballho realizado por Shore e Triassi (1998), foi detectado um potencial de herança bi-parental do DNA plastidial em *Turnera ulmifolia* (Turneraceae). No trabalho de Avni e Edelman (1991), foi realizado um estudo para seleção direta da herança paterna de cloroplasto na progênie sexual de *Nicotiana tabacum* utilizando a sensibilidade e resistência de tentoxina (clorose) como detector de herança paterna. Além dessas espécies foi relatada a herança paterna do cloroplasto em *Pinus* (Brent e David, 1989). Com base nesta última evidência, foi realizado um trabalho por Chen e cols. (2002), no qual eles detectaram herança paterna do cloroplasto em híbridos de *Pinus* utilizando o espaçador intergênico *trn*L-*trn*F e a técnica de PCR–SSCP (single-strand conformation polymorphism). Considerando o que tem sido relatado na literatura, nossos dados sugerem a existência de herança paterna do genoma plastidial nas espécies da seção *Torva* do gênero *Solanum*, no entanto um maior número amostral deve ser utilizado para a comprovação dessa hipótese. Entretanto, o esperado seria que a relação de parentesco entre o possível híbrido e os seus progenitores estaria melhor evidenciada por ITS, por se tratar de um genoma com herança bi-parental. No entanto, essa relação não foi evidenciada, pois em nenhuma das árvores filogenéticas obtidas com o fragmento ITS, observa-se a espécie de morfologia intermediária relacionada com ambos os parentais, apenas com um deles, *S. guaraniticum*.

Para a região ITS, tem sido relatada uma evolução em concerto que deve homogenizar as cópias (seqüências) e, juntas, a amplificação seletiva e evolução em concerto, podem ocultar informações sobre o processo evolutivo de uma espécie, uma vez que em grande parte das vezes a região ITS homogeniza suas cópias conforme o padrão materno (*N. rustica*) ou paterno (*N. tabacum*) (Chase e cols., 2003). Isto explicaria a deficiência do marcador ITS para demonstrar as

interações dos progenitores e os indivíduos de morfologia intermediária neste trabalho. Freitas e Souza-Chies (2003) comentam que os espaçadores da região de ITS apresentam altas taxas de substituição nucleotídicas, capazes de produzir grande variabilidade, porém as divergências observadas entre as linhagens são afetadas pelo processo de evolução em concerto, o qual é responsável pela homogeneização das seqüências *en tandem* do DNA ribossomal.

Um dos debates mais freqüentes entre os sistematas e taxonomistas tem sido a escolha do critério ótimo, isso é, qual o melhor método para análise filogenética. Em geral têm sido utilizados três métodos básicos para estimar a filogenia, incluindo distância, máxima parcimônia e máxima verossimilhança. As relativas vantagens e desvantagens destes métodos vêm sendo debatidas por vários autores (Faith, 1985; Swofford e Olsen, 1990; Kunhner e Felsensteins, 1994; Huelsenbeck, 1995; Farris e cols., 1996; Lewis, 1998; Steel e Penny, 2000; Pol e Siddall, 2001; Goloboff, 2003; Gaucher e Miyamoto, 2005). Geralmente, mais de um método é empregado nas análises filogenéticas para compensar as desvantagens de cada método, tornando as evidências evolutivas mais consistentes. Recentemente, estudos comparativos têm iniciado uma nova proposta de reconstrução filogenética, que corresponde à análise Bayesiana, que apesar de ter sido sugerida por Felsenstein em 1968 (ver Hulsenbeck e cols., 2002), tem se tornado mais amplamente conhecida recentemente, e para a qual programas computacionais têm sido disponibilizados. Algumas revisões recentes sobre análise Bayesiana têm sido propostas por Huelsenbeck e Ronquist (2001), Lewis (2001), Huelsenbeck e cols. (2002), Holder e Lewis (2003), e Ronquist (2004). No presente trabalho foi observada uma semelhança muito forte entre a topologia das árvores obtidas a partir das análises de máxima verossimilhança (maximum likelihood) e inferência Bayesiana, ambas baseadas em métodos probabilísticos. Segundo Archibald e cols. (2003), quando aplicada à reconstrução filogenética, a inferência Bayesiana é similar à máxima verossimilhança (MV), a qual aplica uma função de probabilidade e um modelo determinado de substituição nucleotídicas, apresentando muito das vantagens e desvantagens da MV. Desta forma, é desnecessária a utilização de ambos os métodos, podendo fazer a

escolha por apenas um deles. Um dos benefícios apresentados pela Inferência Bayesiana (relativo à máxima verossimilhança) em filogenia, é a rapidez das análises. Isto se deve à implementação de MCMC (Markov Chain Monte Carlo), que possibilita estimar a probabilidade posterior da distribuição das árvores, o que elimina muito dos cálculos complexos e integrações e, se baseia em comparações de simples estimativas (Huelsenbeck e cols., 2002). Embora a análise Bayesiana seja mais lenta que a máxima parcimônia, ambas são mais rápidas em comparação à máxima verossimilhança. No estudo de Reed e cols. (2002) foi verificada uma diferença de 84 dias entre as análises de Inferência Bayesiana e máxima verossimilhança, as quais apresentaram a mesma topologia das árvores. Estas evidências justificam as topologias semelhantes encontradas entre as árvores probabilísticas no artigo 2 e ressalta as vantagens em se utilizar somente a análise Bayesiana, sobretudo quando o número de táxons empregados no estudo é grande.

Outro fator que alterou a topologia das árvores ao se utilizar a mesma matriz de dados, foi o tratamento dado aos “gaps”, os quais foram tratados como dados faltantes ou como quinto estado de caractere na análise de parcimônia. No artigo 2, observa-se um aumento considerável do número de caracteres filogeneticamente informativos na matriz, que considera os “gaps” como quinto estado de caractere e, em ambos os casos, há um baixo índice de homoplasia. Segundo Giribet e Wheeler (1999), os “gaps” originam-se de eventos biológicos particulares, tais como mutação (deleção/inserção) e, por este motivo, eles podem conter tanta informação histórica como a que é observada com as mudanças nucleotídicas. Além disso, os autores comentam que os métodos que não consideram a informação dos “gaps” podem ser menos explicativos, uma vez que descartam algumas das informações importantes, dificultando a elucidação da verdadeira reconstrução filogenética dos táxons.

A utilização de ambos os genomas plastidial e nuclear, assim como a aplicação de mais de um fragmento por genoma é muito importante para a reconstrução filogenética e determinação da história evolutiva das espécies. Porém, existem dúvidas a respeito de combinar ou não as matrizes de dados.

Vários autores têm discutido sobre as vantagens e desvantagens da análise combinada (De Queiroz e cols., 1995; Miyamoto e Fitch, 1995; Farris e cols., 1995; Huelsenbeck e cols., 1996), enquanto que outros autores comentam que os testes de congruência nem sempre correspondem a uma evidência definitiva, uma vez que não se sabe se é apropriado combinar os dados (Sullivan, 1996; Johnson e Soltis, 1998; Soltis e Soltis, 2000). Nos estudos realizados aqui, foram utilizados dois diferentes testes para verificar o grau de congruência entre as matrizes correspondentes aos marcadores nucleares e plastidiais (PHT (partition homogeneity test ou incongruence lenght test – ILD) e PBS (partition Bremer suport)). Observou-se que as diferenças nas escalas taxonômicas, tratadas nos dois artigos, afetaram diretamente o resultado dos testes de congruência entre as seqüências utilizadas. Em ambos os artigos oi verificada uma significante incongruência entre os marcadores plastidiais e nucleares quando utilizado o PHT. Entretanto, estes resultados só foram corroborados com os de PBS no artigo 1, onde foram incluídas na análise várias espécies oriundas de outras seções do subgênero *Leptostemonum* de *Solanum*. A incongruência observada em ambos os testes (artigo 1) pode ter sido ocasionada pela falta de homogeneidade nas seqüências entre os táxons, devido ao grande número de mutações (deleções/inserções), e pelas altas taxas de homoplasias apresentadas pela região de ITS. Entretanto, no artigo 2, o teste de PBS não corroborou com os resultados de PHT, mostrando uma congruência entre as regiões de cloroplasto e nucleares, com os índices de PBS não mostrando discrepâncias entre eles para construção dos clados na análise combinada. No artigo 2 foram utilizados apenas representantes da seção *Torva* de *Solanum* no grupo interno, os quais apresentaram uma homogeneidade nas seqüências, com poucas mutações, e ambos marcadores apresentaram taxas de homoplasias muito baixas, o que leva a dar mais credibilidade ao observado com o teste de PBS.

No artigo 2, apesar de ITS mostrar uma das amostras de *S. adspersum* fora do grupo formado pelos demais indivíduos dessa espécie, esta variação não ultrapassou a barreira específica, e portanto não invalida seu uso como marcador filogenético, uma vez que a maioria dos indivíduos de uma mesma espécie

formam clados com alto suporte. A incongruência observada pelo PHT pode ser igualmente explicada pelo tamanho das partições das regiões de cloroplasto (1848 pb) e nuclear (691pb), como discutido no artigo 2, uma vez que este tipo de teste pode ser sensível quando os conjuntos de dados diferem muito em relação ao tamanho (Dowton e Austin, 2002).

Heterogeneidades significantes entre os grupos de dados podem impedir sua subseqüente combinação; porém, tem sido mostrado que o PHT é especialmente sensível a mutações silenciosas e diferentes taxas de mutação entre os grupos de dados, e isto pode representar uma medida tendenciosa e incorreta de congruência (Barker e Lutzoni, 2002; Darlu e Lecointre, 2002; Dolphin e cols., 2000). Portanto, em nosso trabalho optamos em realizar análises separadas e combinadas, e compararmos os resultados das topologias das árvores filogenéticas. Isto também foi realizado no trabalho de Neves e cols. (2005) em que eles observaram uma contrastante taxa evolutiva entre as regiões de ITS e *trn*T-*trn*F, optando em combinar os dados mesmo com a incongruência observada entre as partições, por PHT.

A técnica de ISSR mostrou-se útil para avaliar o grau de divergência genética entre as espécies da seção *Torva* de *Solanum.* Com o uso desses marcadores foi possível detectar grupos de espécies mais relacionadas nas análises de distância e de similaridade, em adição aos outros marcadores utilizados neste trabalho. Os quatro “primers” utilizados para ISSR se mostraram polimórficos e filogeneticamente informativos para os 22 acessos analisados, apesar do alto índice de homoplasias apresentado para este tipo de marcador. O aumento no número de “primers” e de acessos possivelmente melhoraria a resolução no dendograma, agrupando as espécies da seção *Torva* em um único grupo, afastando a hipótese de agrupamento por distribuição geográfica, levantada no artigo 2. Entretanto, para ISSR o número de “primers” utilizado para análises não necessariamente necessita ser elevado. Gilbert e cols. (1999) utilizou apenas dois “primers” para distinguir 37 acessos estudados. Similarmente, quatro “primers” foram suficientes para distinguir 34 cultivares de batata (Prevost e

Wilkinson, 1999) e três “primers” puderam distinguir 16 genótipos de *Ribes rubrum* (Lanham e Brennan, 1998).

Entretanto, o potencial oferecido pelos marcadores ISSR depende da variedade e freqüência dos microssatélites, variando com as espécies e com os motivos SSR que são utilizados como “primer” (Morgante e Olivieri, 1993; Depeiges e cols., 1995). Sendo o “primer” um SSR, a freqüência e distribuição dos microssatélites em diferentes espécies também influenciam a geração de bandas. Na análise de ISSR, a espécie *S. adspersum* foi a que apresentou o maior número de bandas, mostrando-se a mais distante entre as espécies da seção *Torva*, fato observado nas análises de similaridade e das coordenadas principais. Isto se deve, provavelmente, aos “primers” utilizados nesta análise e à freqüência de microssatélites, apresentada por esta espécie. É provável que um maior número de “primers” oferecesse uma informação adicional, uma vez que poderia atingir com maior abrangência o genoma das demais espécies da seção *Torva* de *Solanum*.

6.- Conclusões e Perspectivas

Os estudos realizados nesse trabalho evidenciaram as relações de parentesco entre as espécies da seção *Torva* de *Solanum* e da seção *Torva* com outras espécies do subgênero *Leptostemonum*. Tais dados certamente auxiliarão em trabalhos futuros a serem realizados com outras espécies pertencentes a esta seção, sobre a história evolutiva das espécies da seção *Torva*.

A monofilia da seção *Torva* evidenciada certamente não pode ser estendida a toda seção, uma vez que utilizamos apenas amostras presentes principalmente na região sul do Brasil e uma amostra típica da Argentina e Uruguai (*S. bonariense*). Para a confirmação da monofilia da seção *Torva* seria necessária a inclusão de representantes da seção presentes em outros pontos de dispersão pelo mundo, o que poderá vir a ser feito em trabalhos futuros.

O número de marcadores utilizados e os métodos de inferência filogenética aplicados mostraram-se adequados para evidenciar o grau de parentesco entre as espécies da seção *Torva* na região sul do Brasil. Entretanto, são necessários trabalhos mais aprofundados para afirmar a existência de um híbrido natural entre as espécies *S. guaraniticum* e *S. paniculatum*.

Uma das alternativas para trabalhos futuros que auxiliariam a elucidação deste relacionamento seria a utilização da citogenética em conjunto com a técnica de citometria de fluxo, as quais têm potencial para avaliar o nível de ploidia das espécies e contribuir desta forma para evidenciar a presença de híbridos entre as espécies da seção *Torva* de *Solanum*. A técnica de citometria de fluxo já vem sendo testada por nosso grupo de pesquisa, no entanto, as espécies da seção *Torva* têm se mostrado de extrema dificuldade para esse tipo de técnica. Dois motivos cruciais para o sucesso na utilização de citometria de fluxo dizem respeito à necessidade de se obter material fresco e o período de floração. Existe uma grande quantidade de alcalóides presentes nas folhas destas espécies, o que dificulta a obtenção de um material livre de impurezas. Portanto, para a realização deste trabalho é necessário esperar o período de floração das espécies, e manter os acessos em casa de vegetação para obtenção do material fresco, uma vez que elas estão distribuídas em diferentes pontos no estado do Rio Grande do Sul.

Nosso grupo também vem realizando, em colaboração com o laboratório da Profa. Anete Pereira de Souza da Unicamp, a construção de um banco enriquecido em microssatélites para a espécie *S. paniculatum.* Até o momento, este trabalho nos proporcionou a obtenção de 66 clones que evidenciaram a presença de microssatélites, dos quais 48 foram seqüenciados. As seqüências apresentaram 39 diferentes motivos para os quais estão sendo sintetizados “primers”. Tais “primers” serão testados quanto ao grau de polimorfismo dentro da espécie *S. paniculatum*, e conseqüentemente poderá ser estendido para a determinação de polimorfismo em toda a seção *Torva*.

Além disso, este trabalho contribuirá para estudos futuros com marcadores ISSR que tenham como objetivo testar o grau de variabilidade genética intraespecífica; e estudos que relacionem filogenia e metabólitos secundários, os

quais vêm sendo amplamente realizados com outras espécies da família Solanaceae.

Contudo, as diferentes propostas empregadas neste trabalho, certamente contribuíram para evidenciar a história evolutiva dos representantes analisados da seção *Torva* de *Solanum.* Além disso, auxiliarão pesquisas futuras sobre estes importantes recursos naturais.

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8. – Anexos

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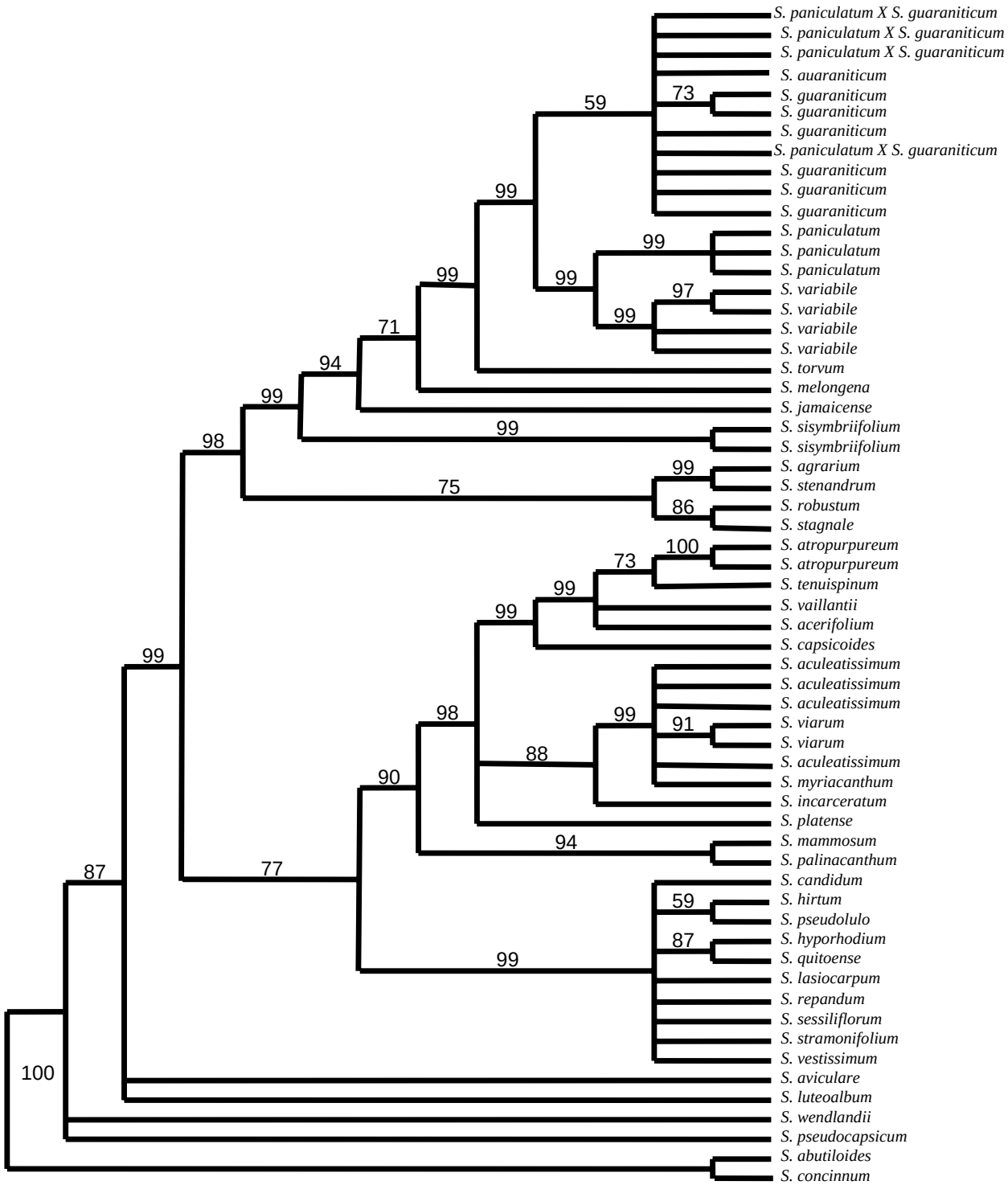
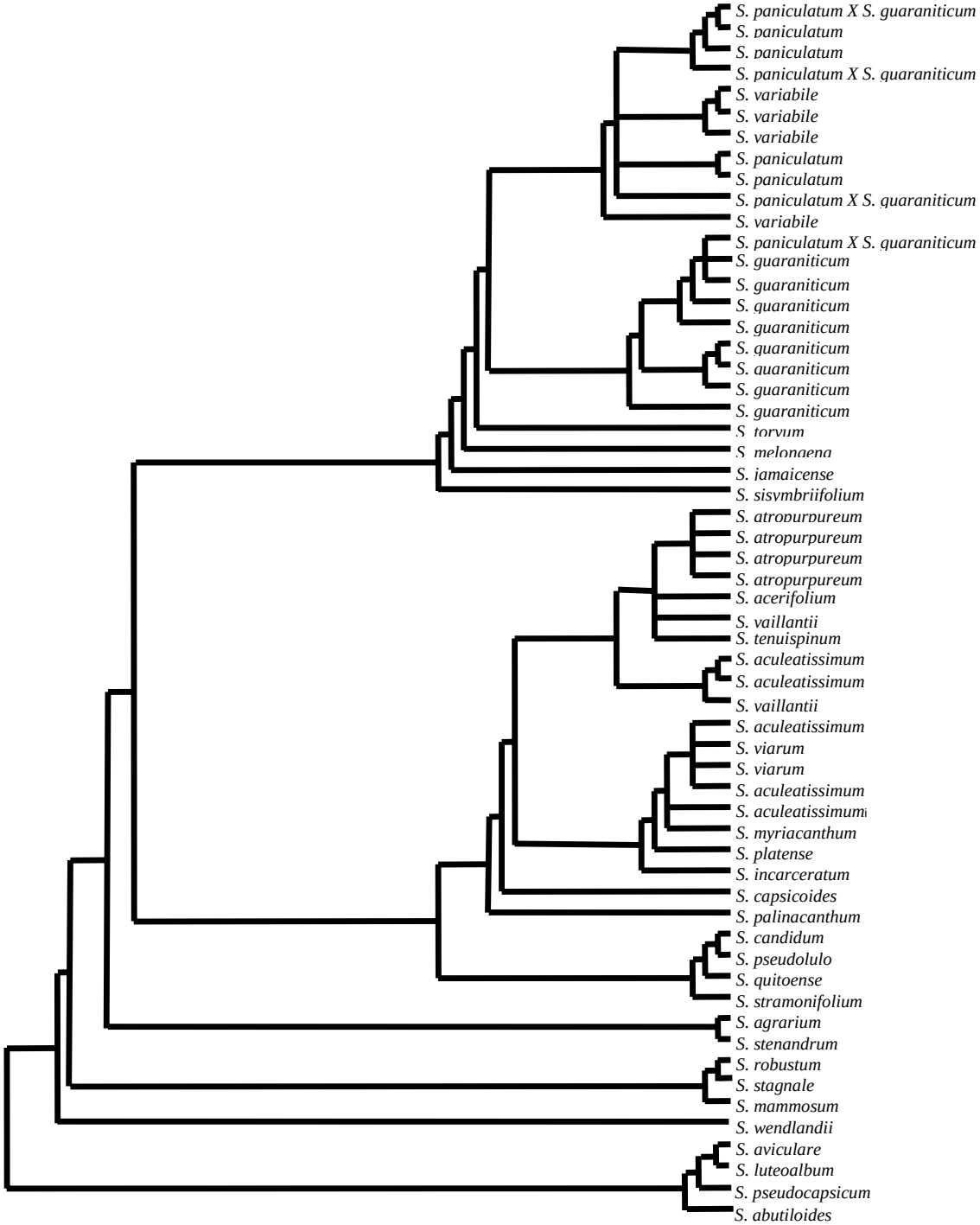
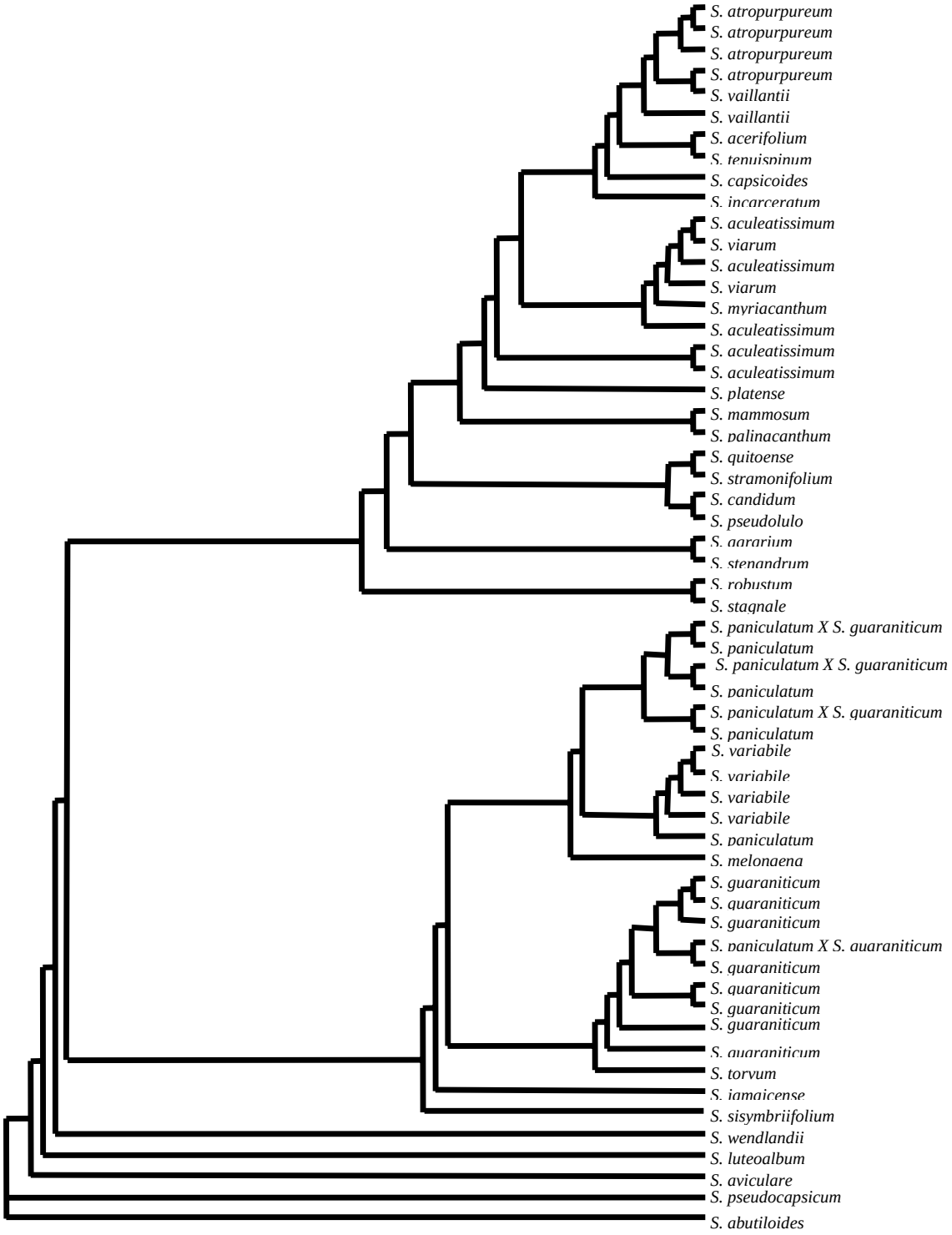


Anexo 1: Árvore filogenética da análise Bayesiana da matriz de ITS (Artigo1).

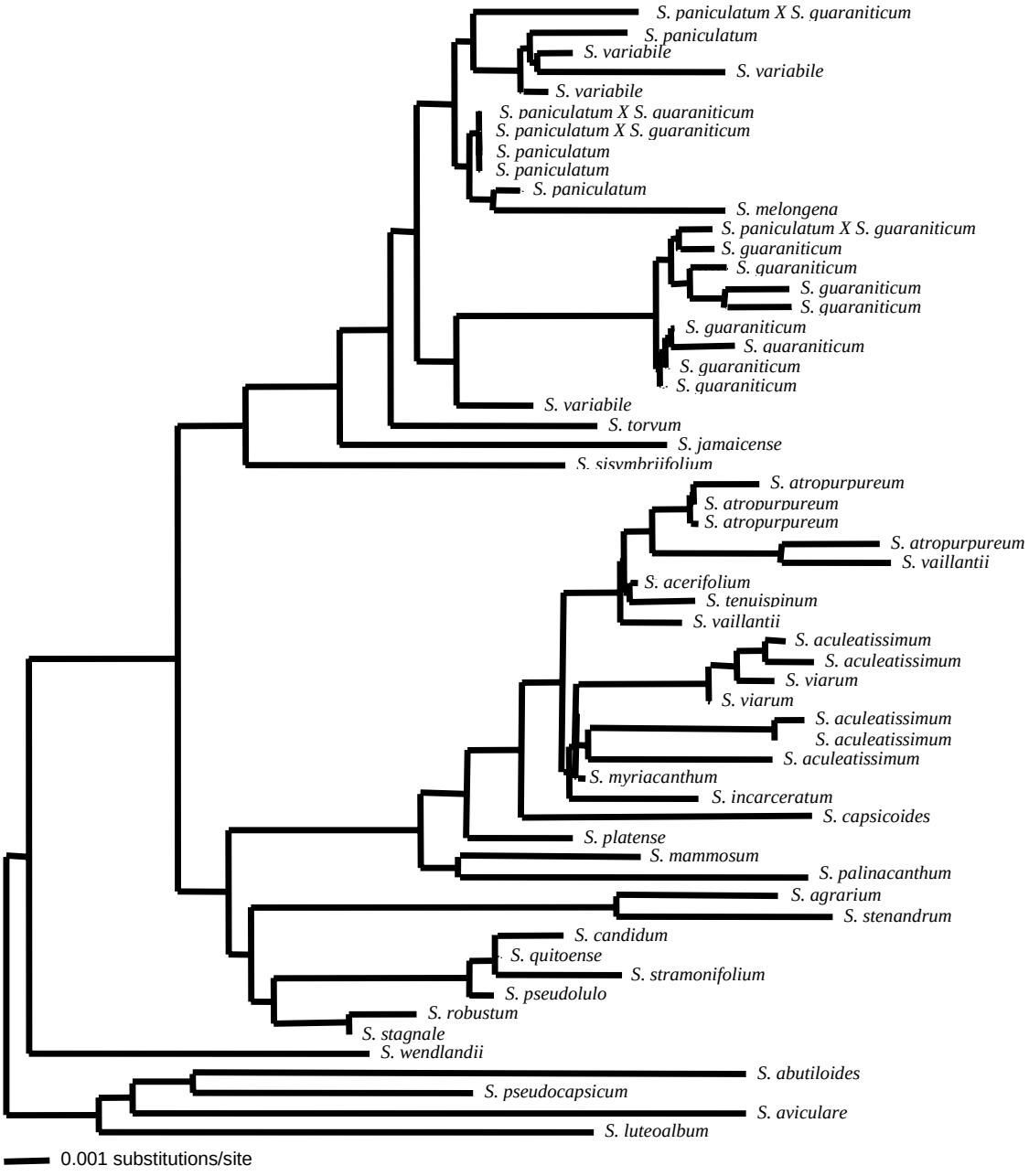
Anexo 2: Árvore filogenética da análise de distância da matriz de ITS (Artigo 1).



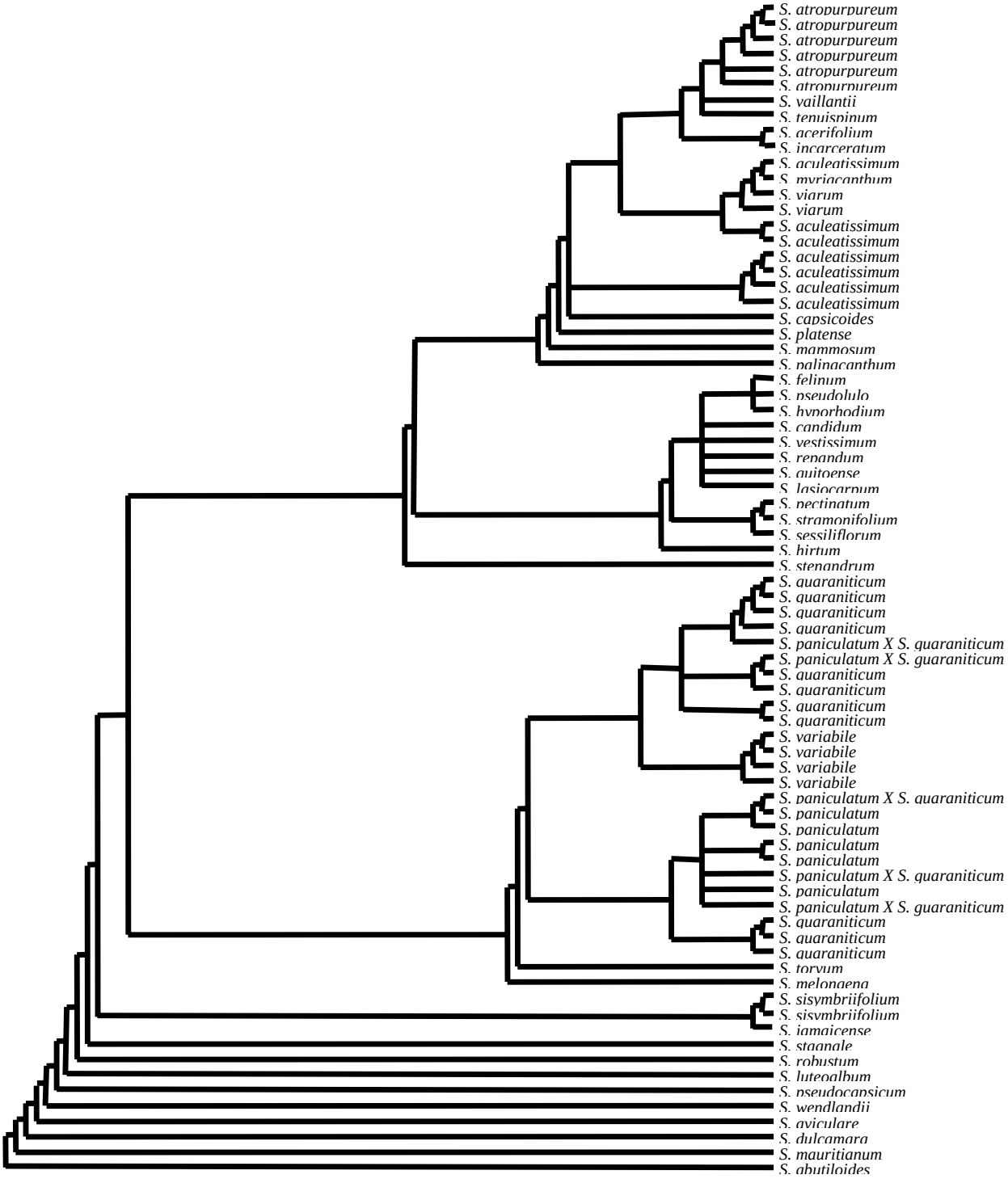
Anexo 3: Árvore consenso da maioria da análise de parcimônia da matriz de *trn*S-*trn*G (Artigo1).



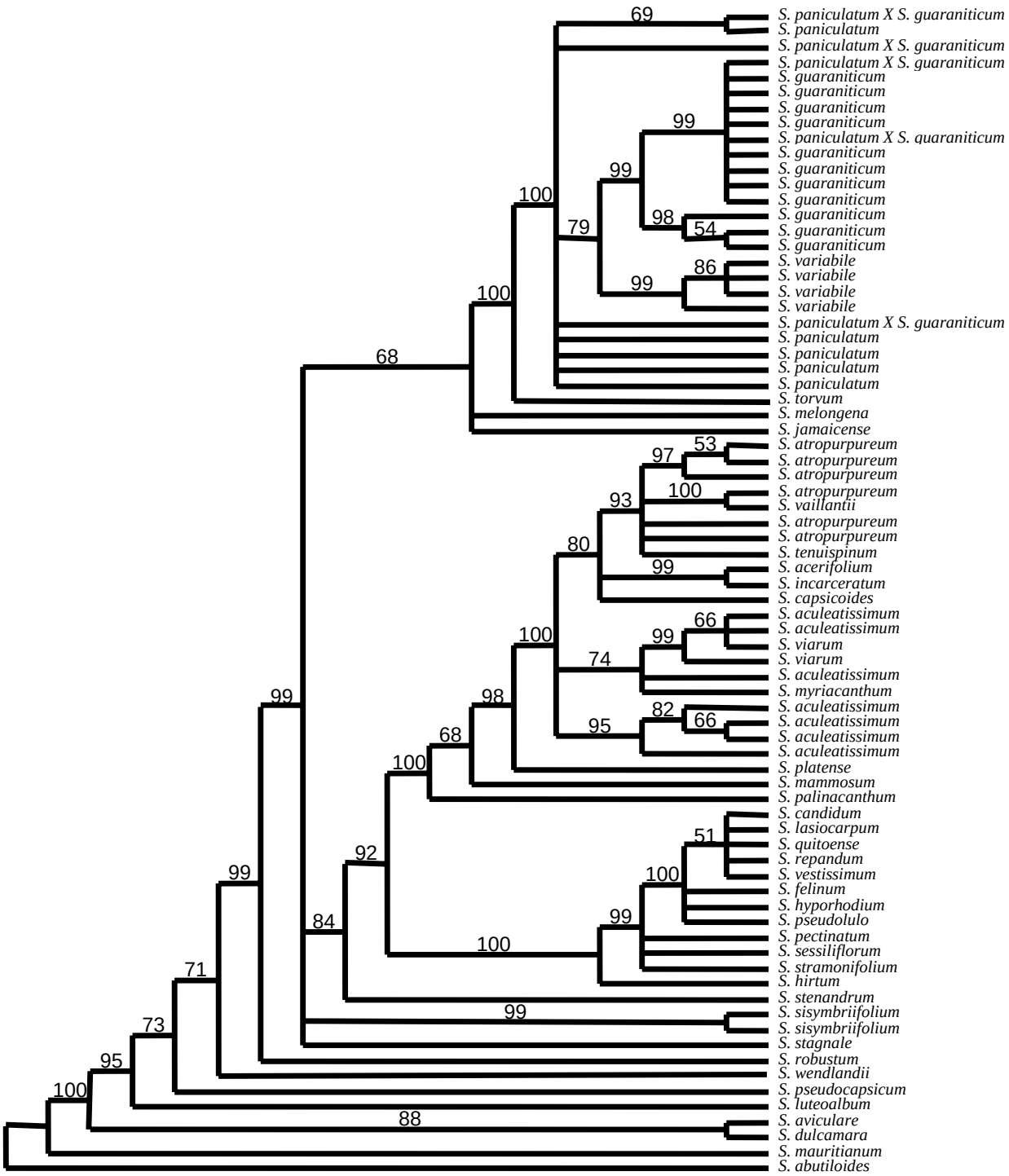
Anexo 4: Árvore filogenética da análise Bayesiana da matriz de *trn*S-*trn*G (Artigo1).



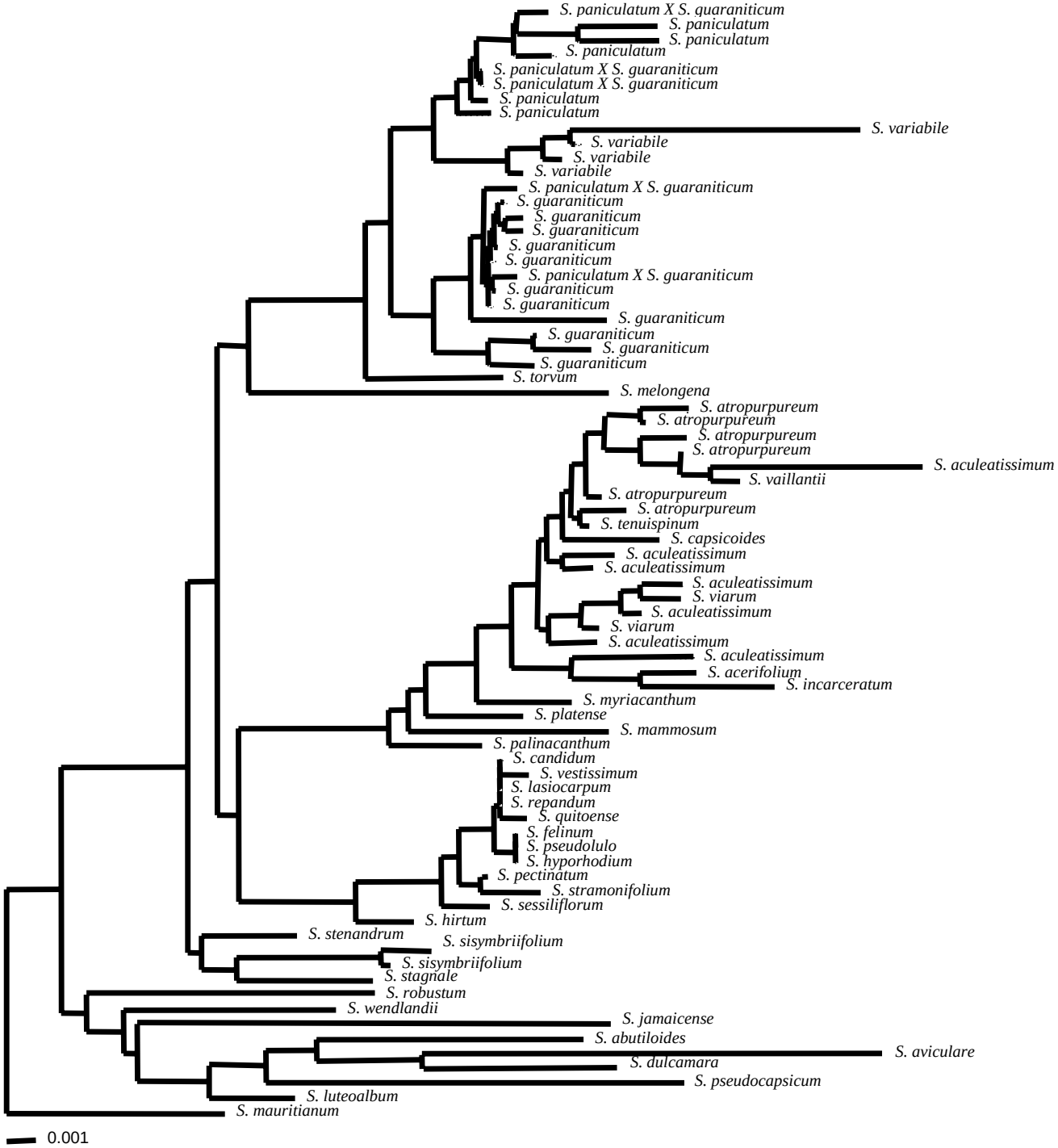
Anexo 5: Árvore filogenética da análise de distância (NJ) da matriz de *trn*S-*trn*G (Artigo 1).



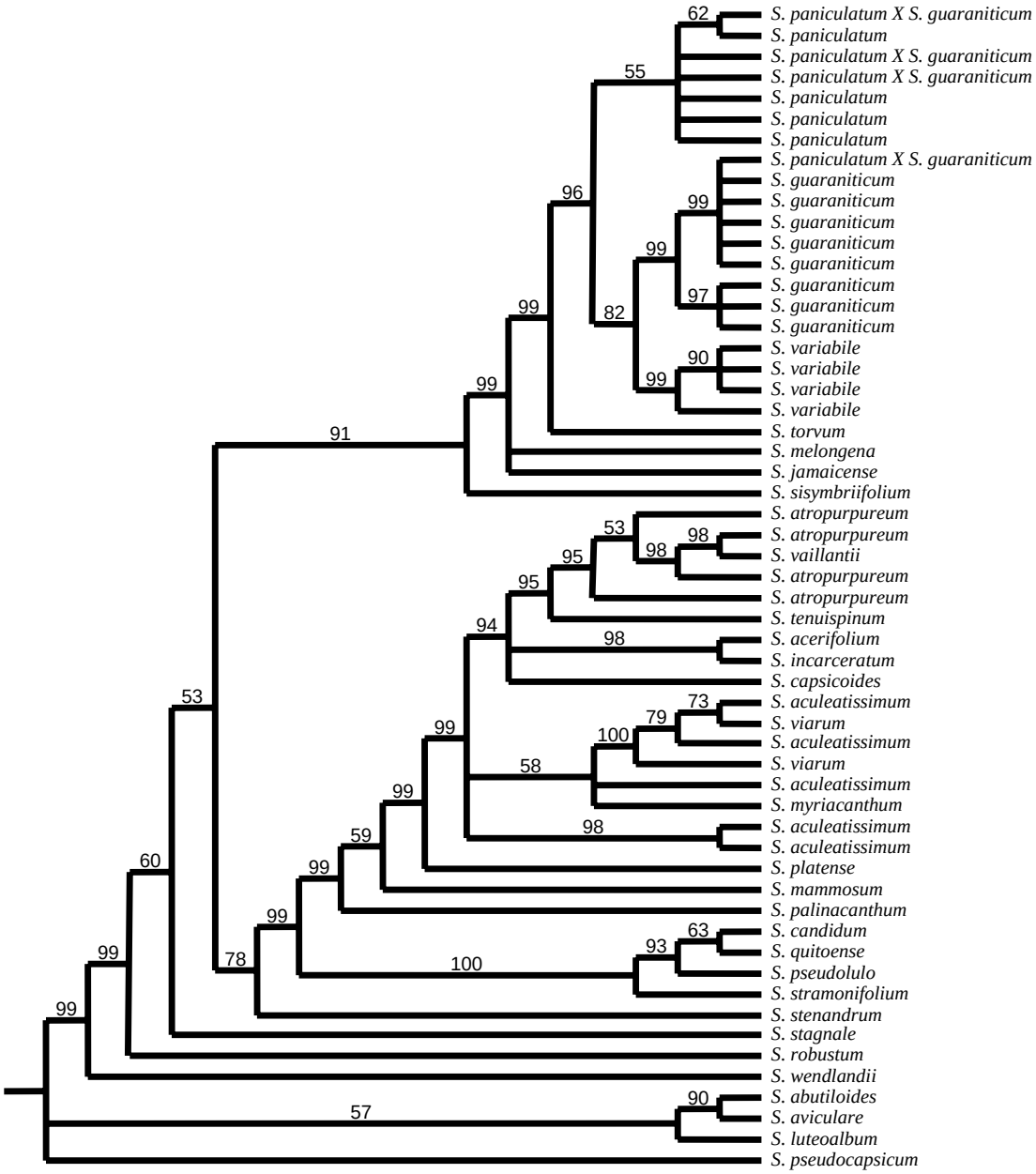
Anexo 6: Árvore consenso da maioria da análise de parcimônia da matriz de *trn*L – *trn*F + íntron *trn*L (Artigo1).



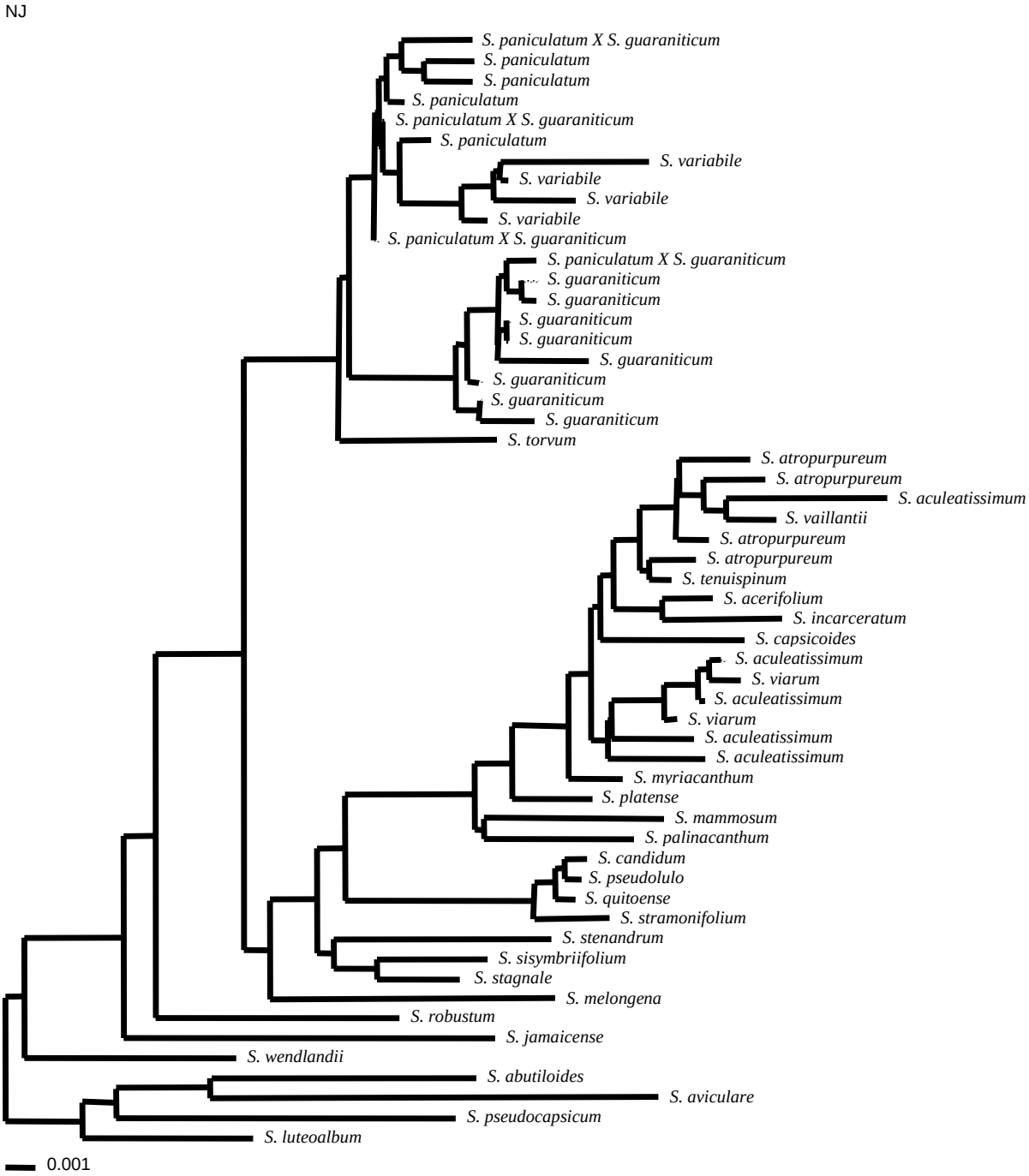
Anexo 7: Árvore filogenética da análise Bayesiana da matriz de *trn*L– *trn*F + íntron *trn*L (Artigo1).



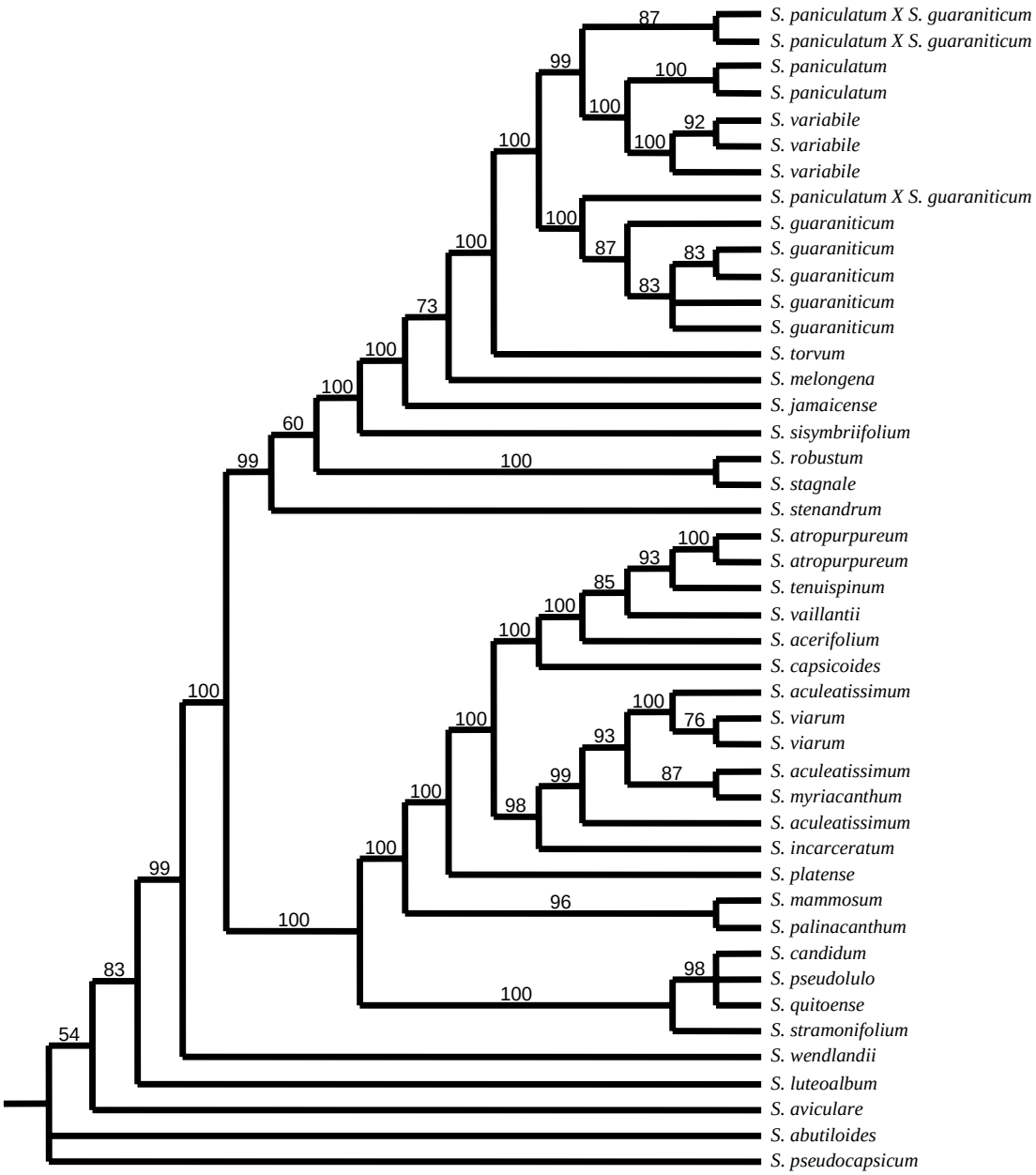
Anexo 8: Árvore filogenética da análise de distância (NJ) da matriz de *trn*L–*trn*F + íntron *trn*L (Artigo1).



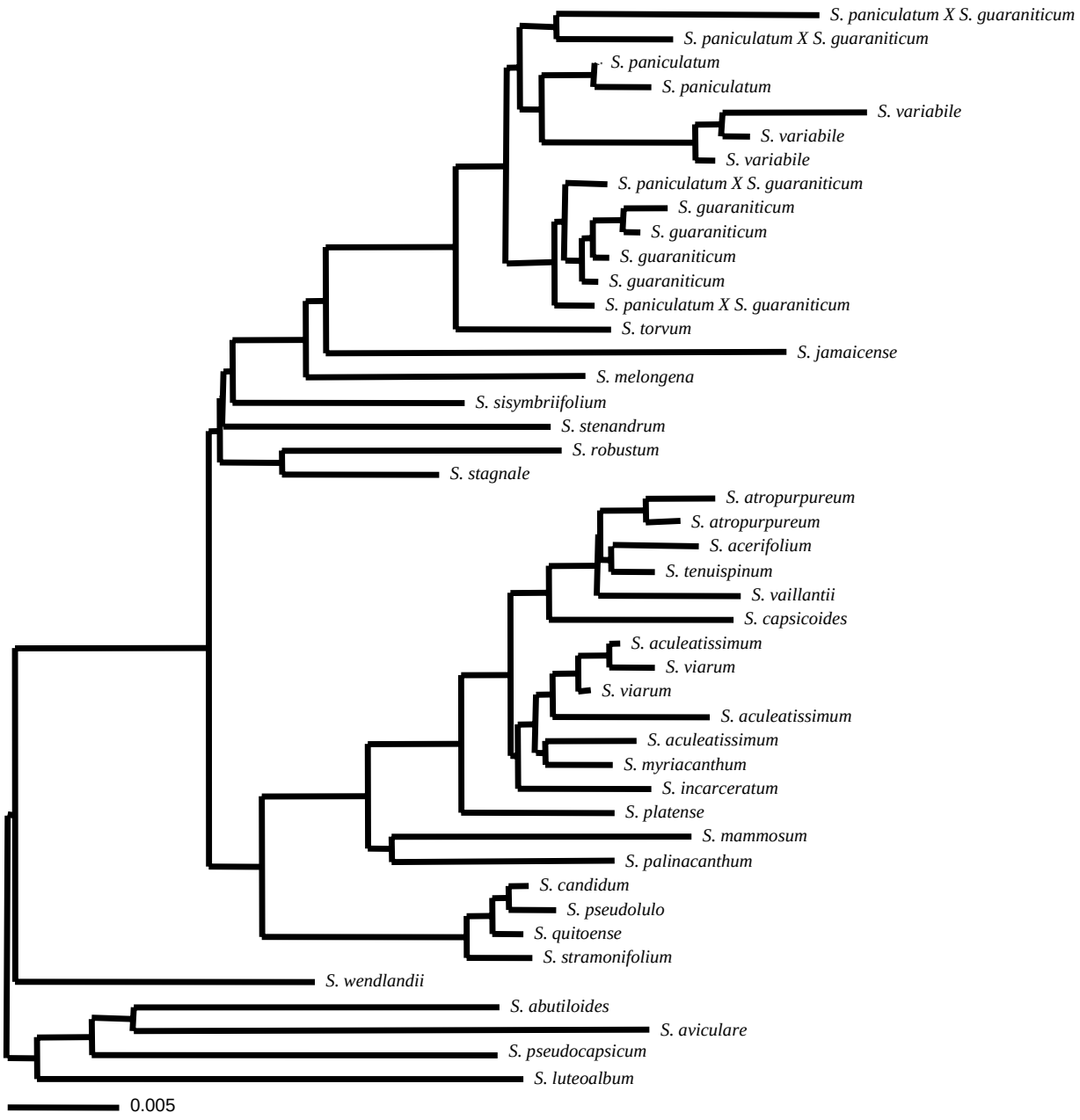
Anexo 9: Árvore filogenética da análise Bayesiana da matriz combinada de cloroplastos (Artigo 1).



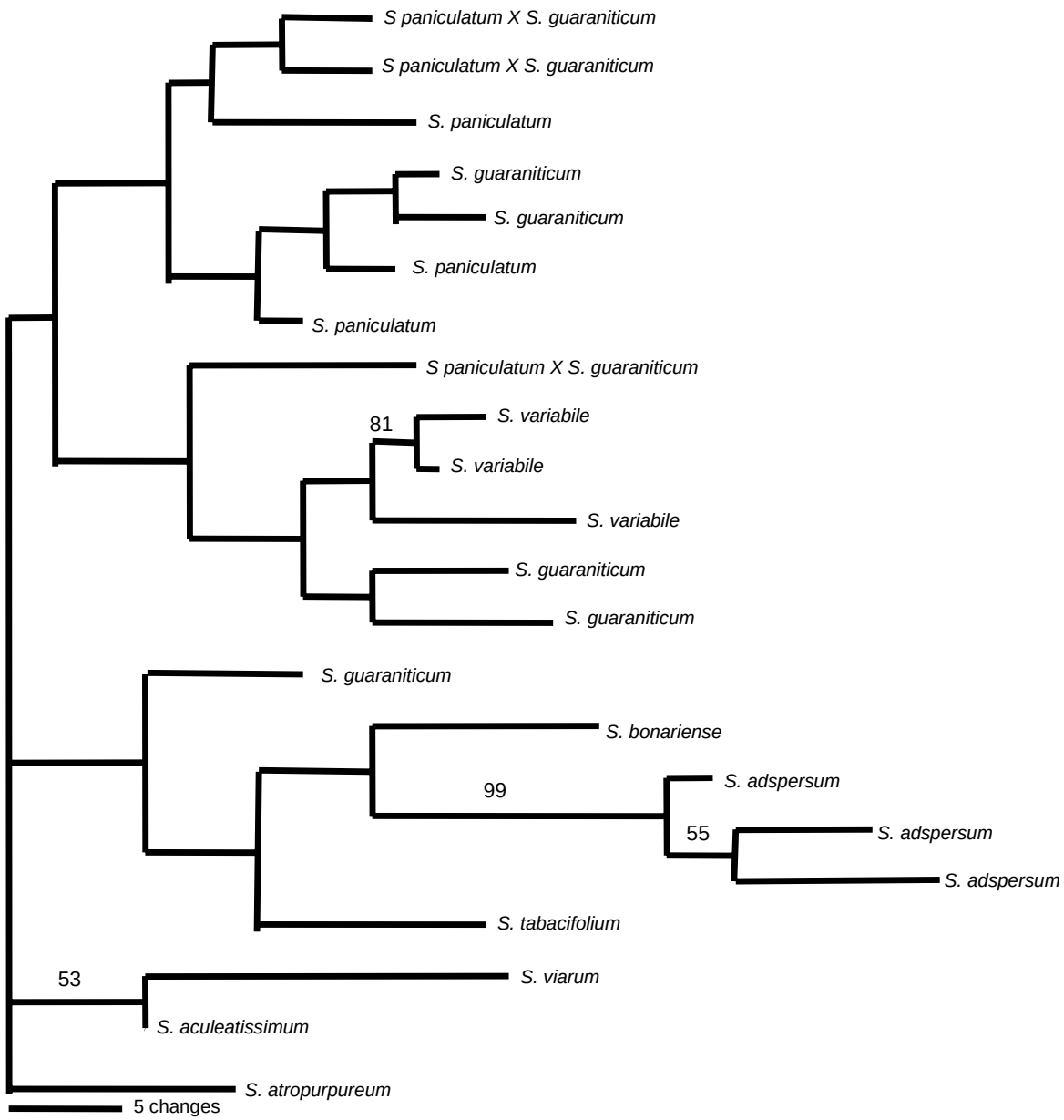
Anexo 10: Árvore filogenética da análise de distância da matriz combinada de cloroplastos (Artigo 1).



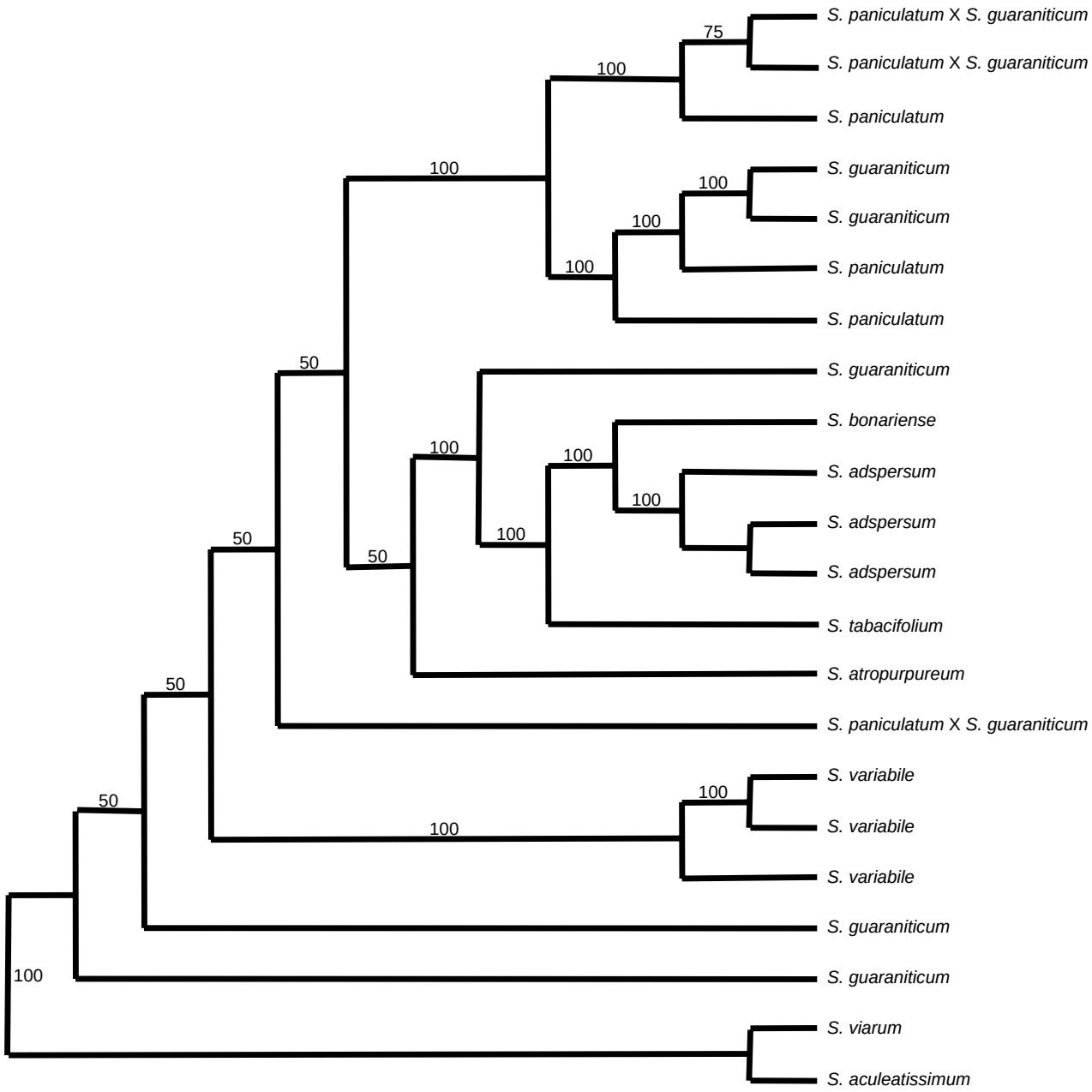
Anexo 11: Árvore filogenética da análise Bayesiana da matriz combinada dos quatro fragmentos (Artigo 1).



Anexo 12: Árvore filogenética da análise de distância da matriz combinada dos quatro fragmentos (Artigo 1).

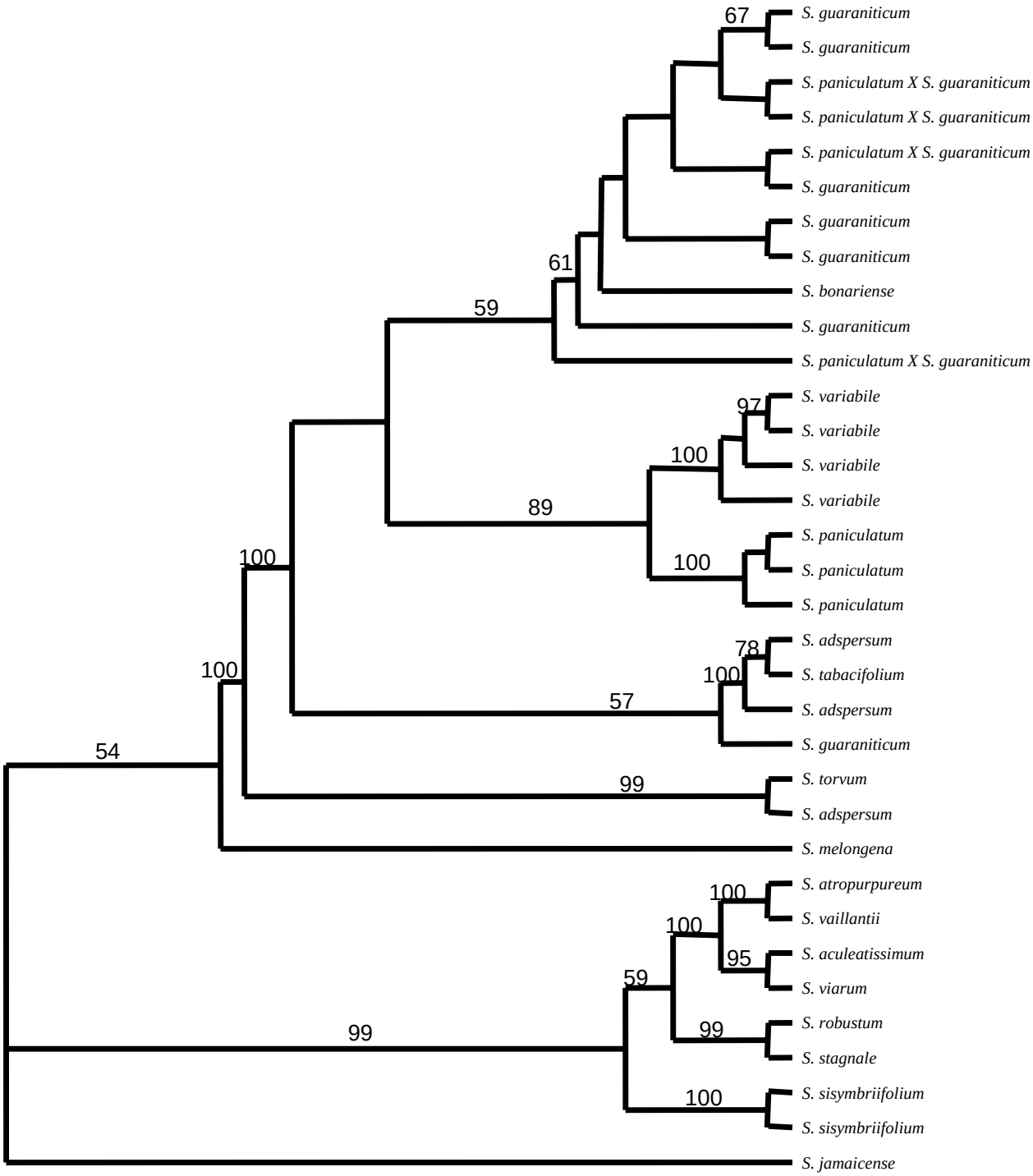


Anexo 13: Uma das quatro árvores da análise de parcimônia obtida com a matriz de dados de ISSR (Artigo 2).



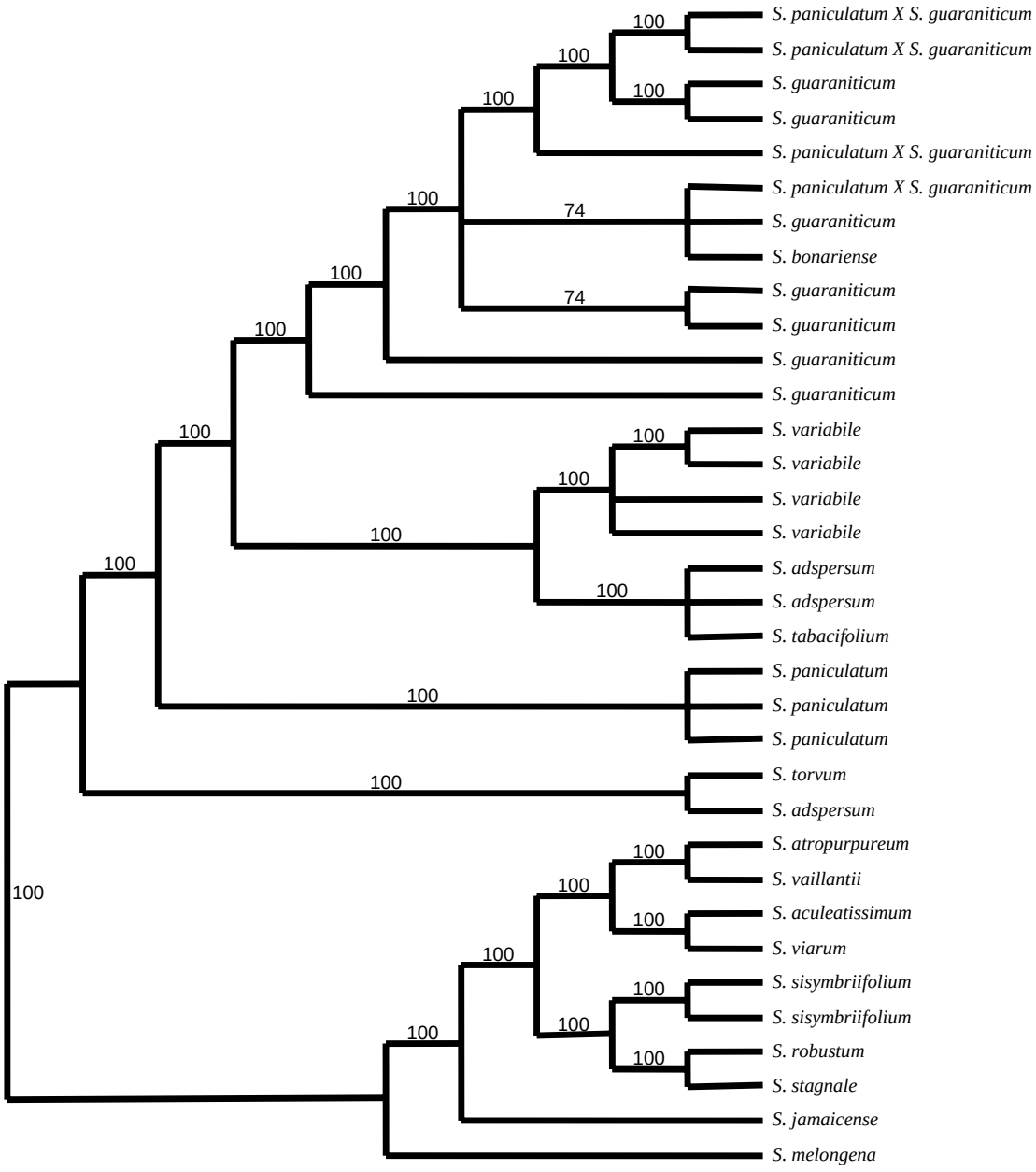
Anexo 14: Árvore consenso da maioria da análise de parcimônia obtida com a matriz de dados de ISSR (Artigo 2).

Anexo 15: Árvore filogenética da análise de máxima verossimilhança da matriz de ITS (Artigo 2).



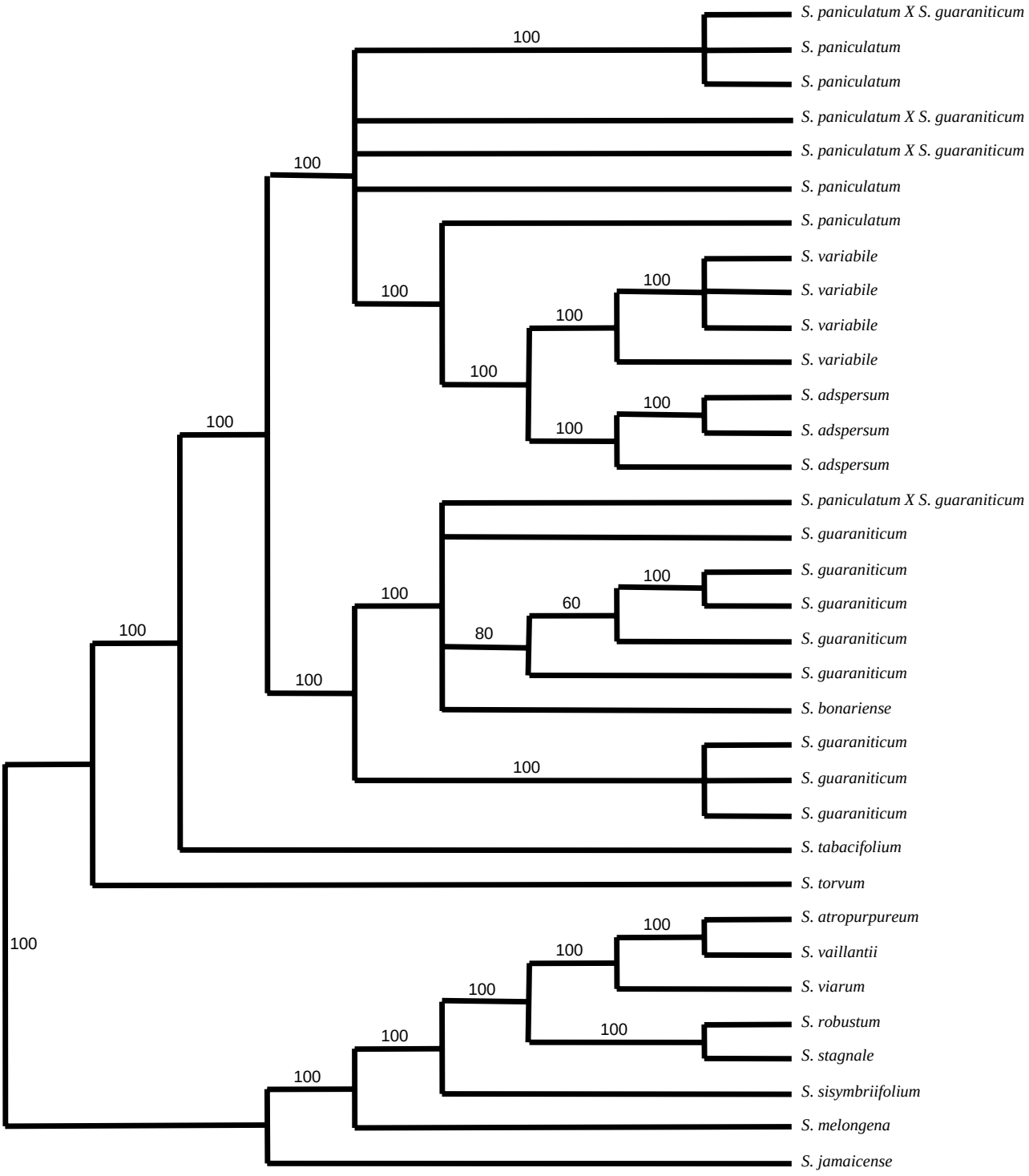
Anexo 16: Árvore filogenética da análise Bayesiana da matriz de ITS (Artigo 2).

Anexo 17: Árvore consenso da maioria da análise de parcimônia da matriz de ITS com os índels tratados como quinto estado de caractere (Artigo 2).

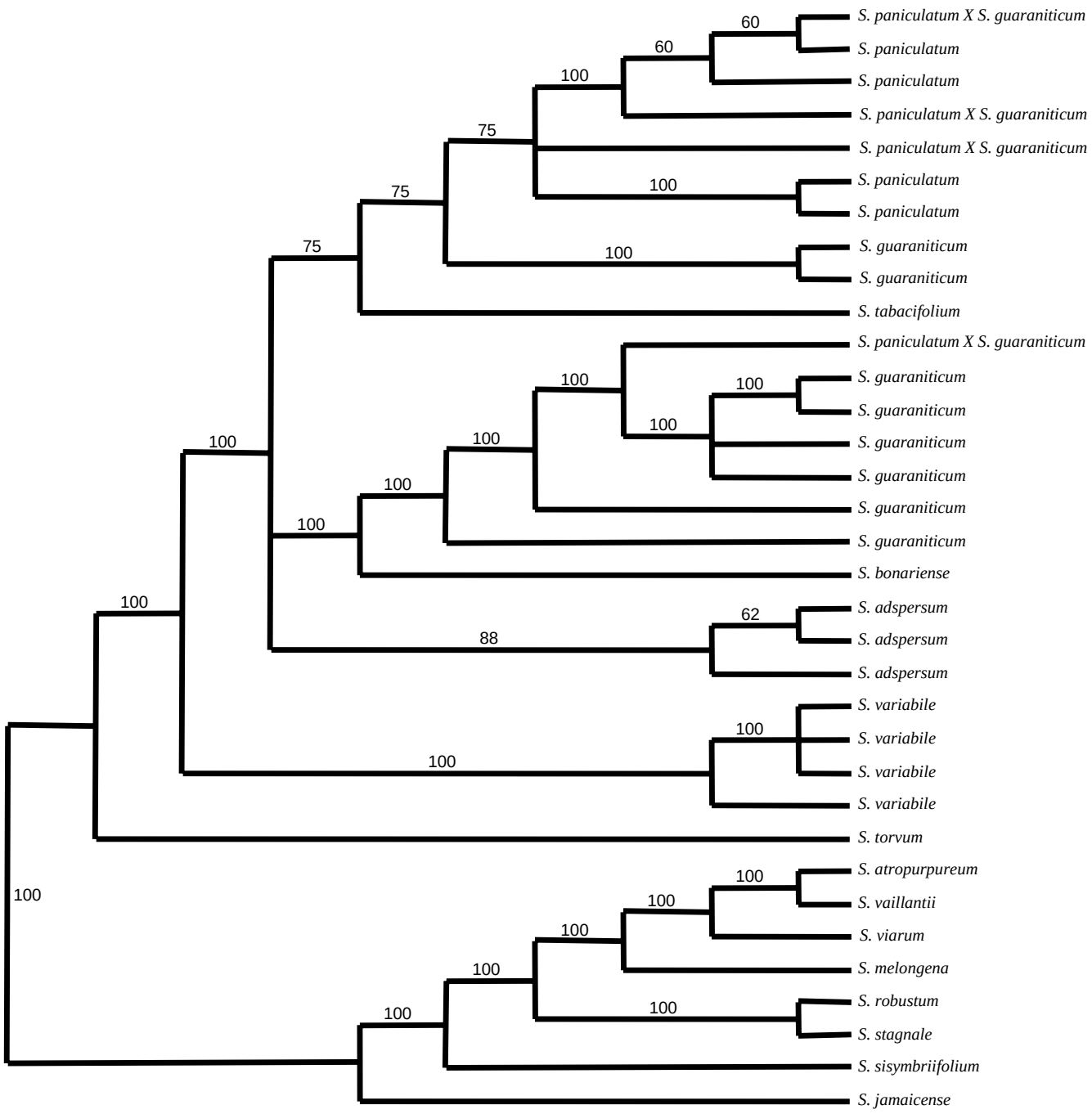


Anexo 18: Árvore consenso da maioria da análise de parcimônia da matriz de ITS com os índels tratados como dados faltantes (Artigo 2).

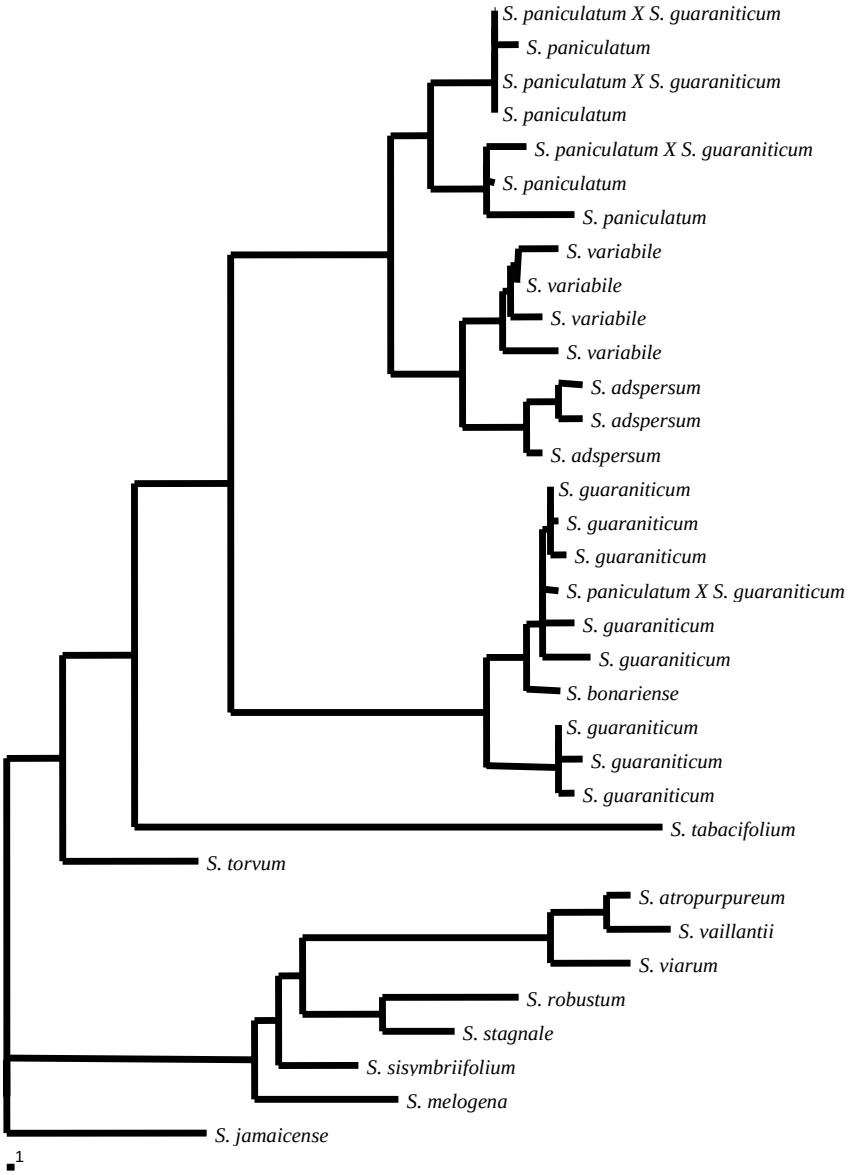
Anexo 19: Árvore filogenética da análise de distância da matriz de ITS (Artigo 2).



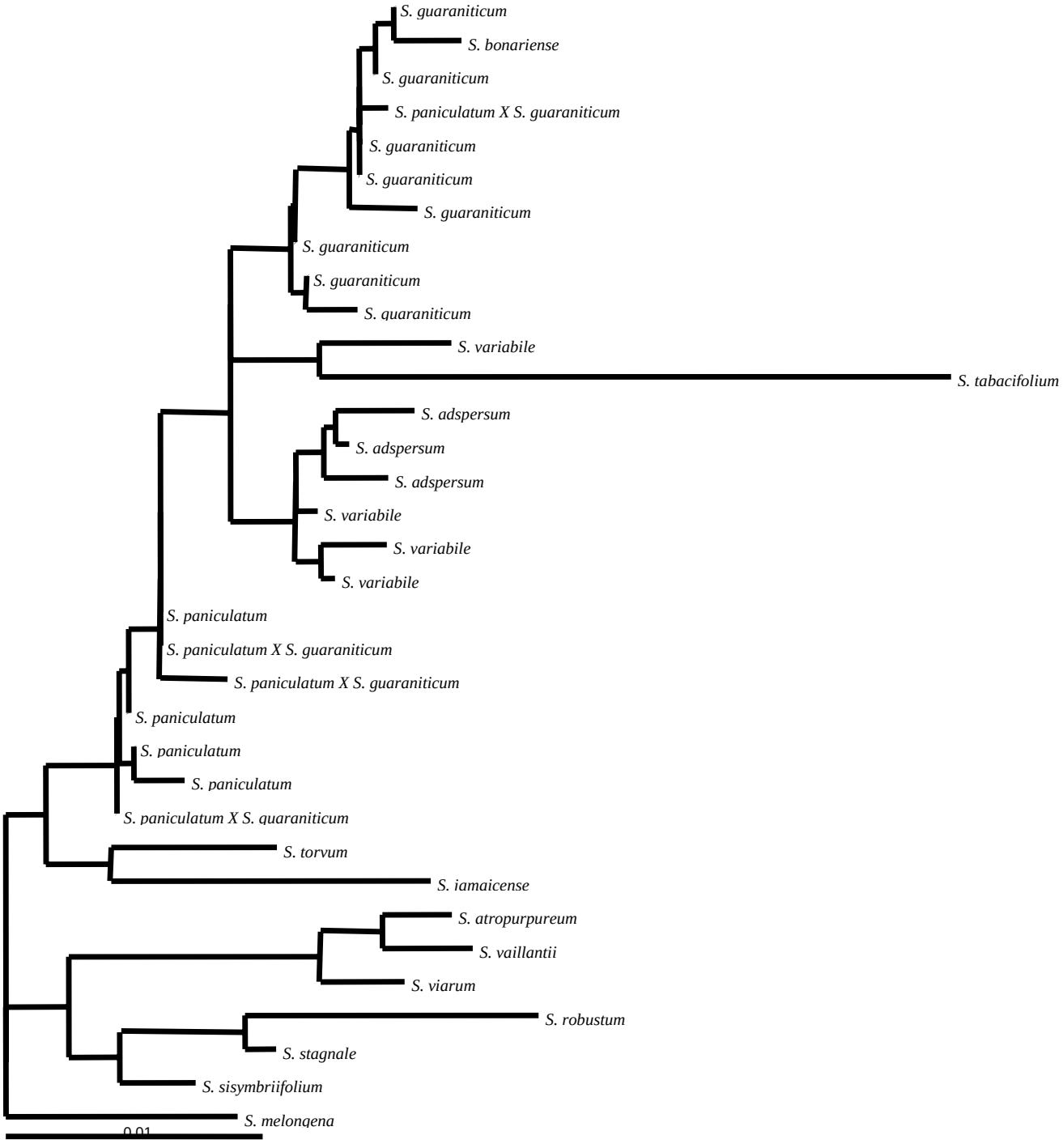
Anexo 20: Árvore consenso da maioria da análise de máxima verossimilhança da matriz de cloroplasto (Artigo 2).



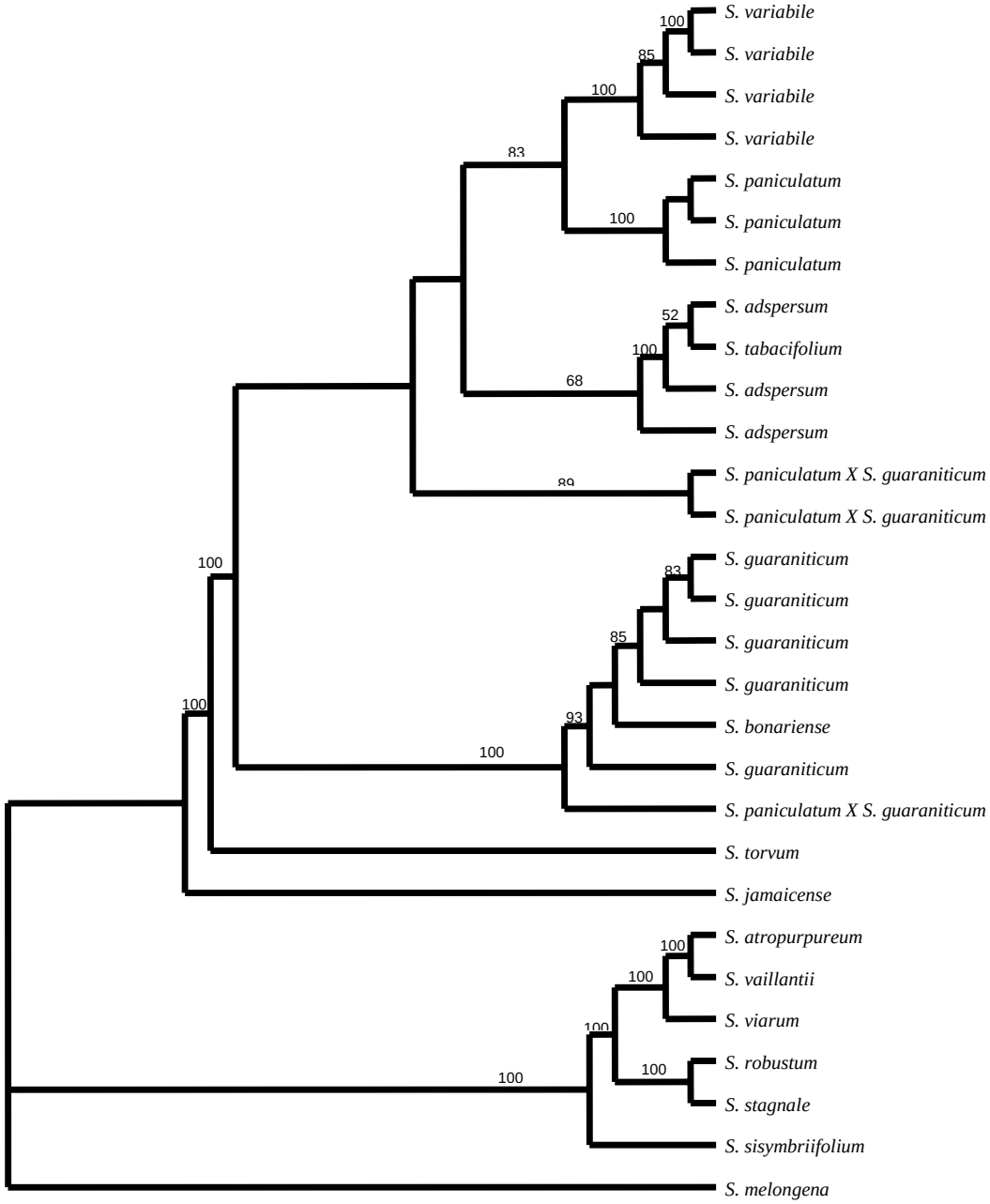
Anexo 21: Árvore consenso da maioria da análise de parcimônia da matriz de cloroplasto, com os índels tratados como quinto estado de caractere (Artigo 2).



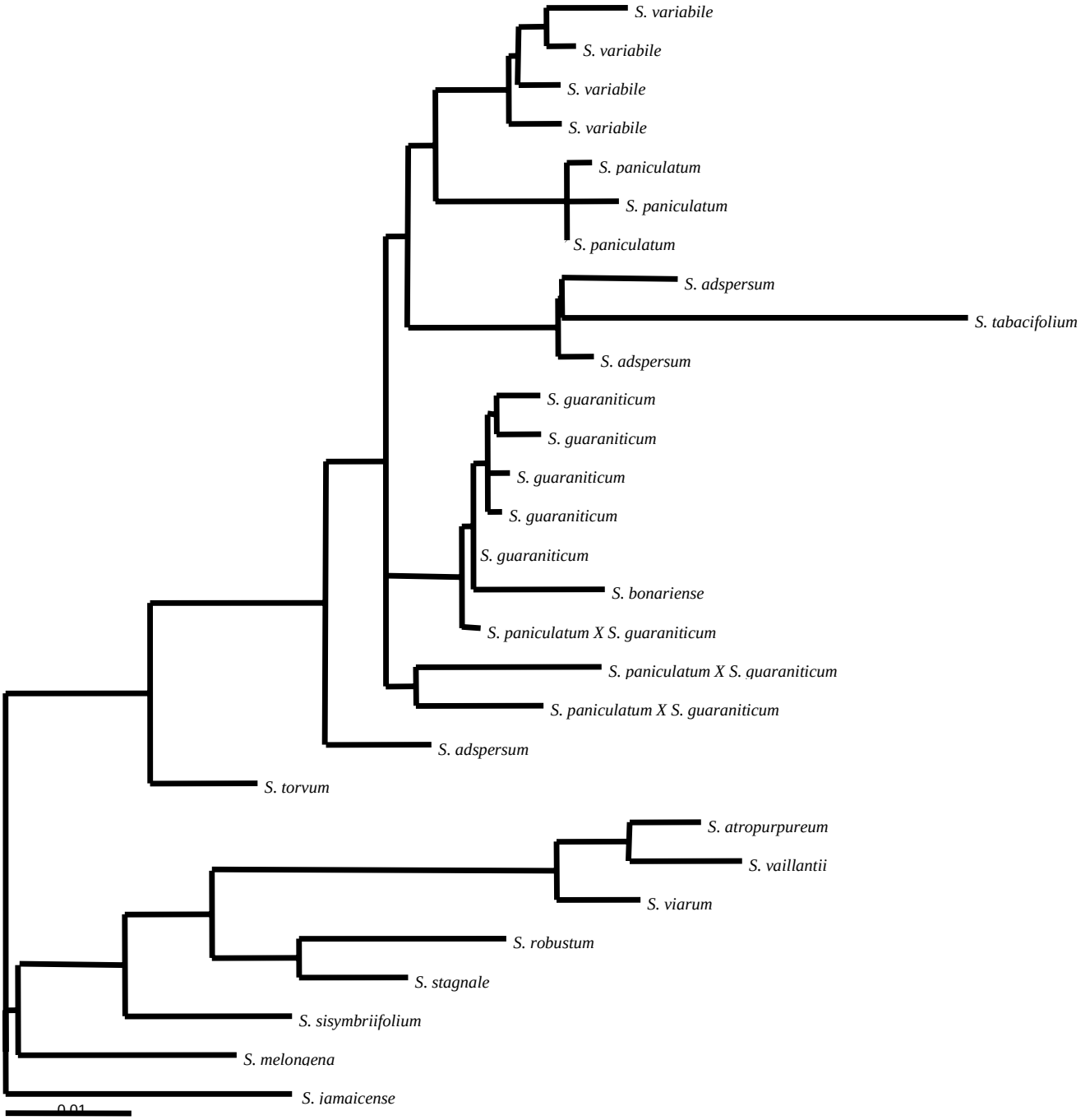
Anexo 22: Árvore consenso da maioria da análise de parcimônia da matriz de cloroplasto, com índels tratados como dados faltantes (Artigo 2).



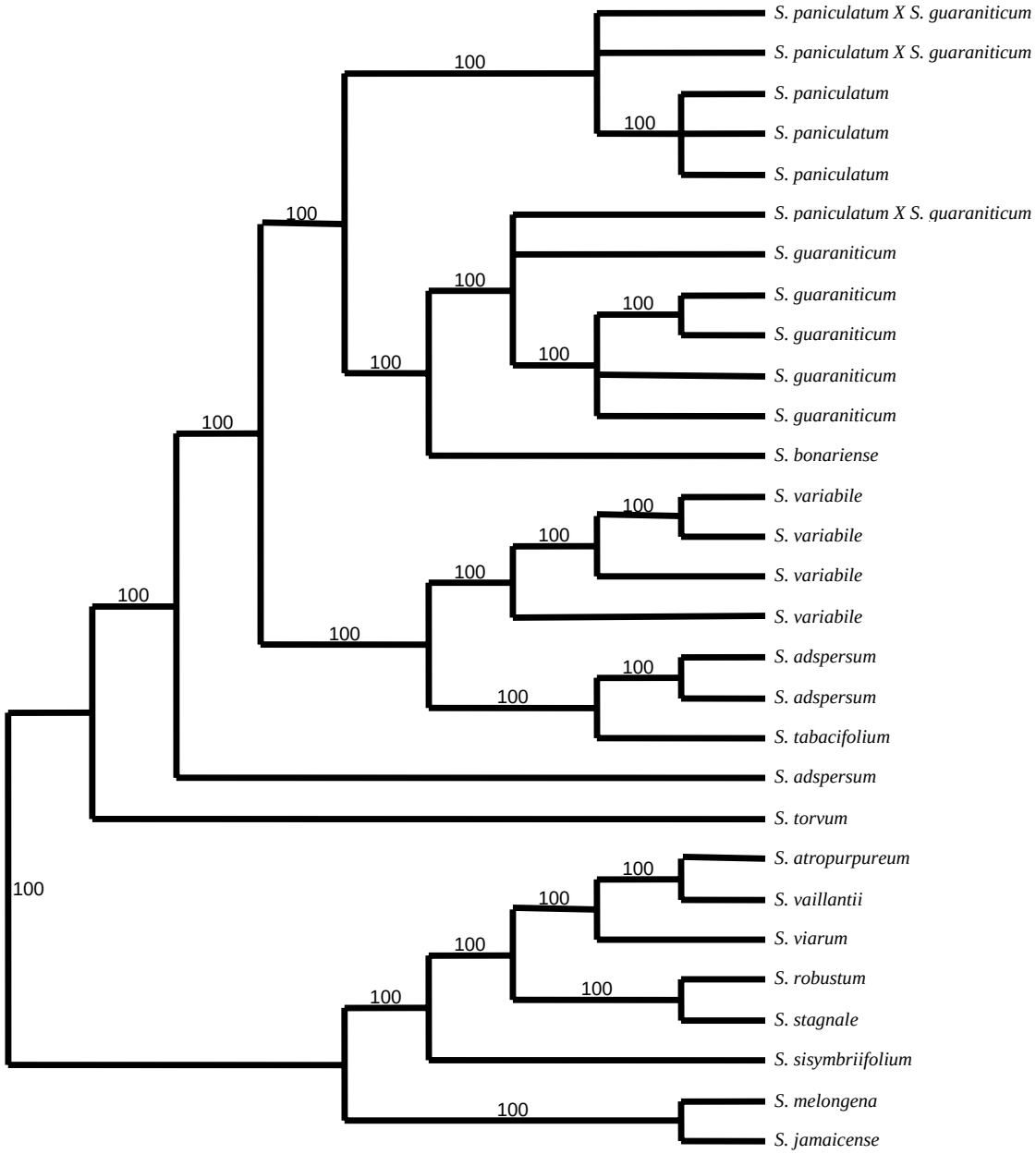
Anexo 23: Árvore filogenética da análise de distância obtida com matriz de cloroplasto (Artigo 2).



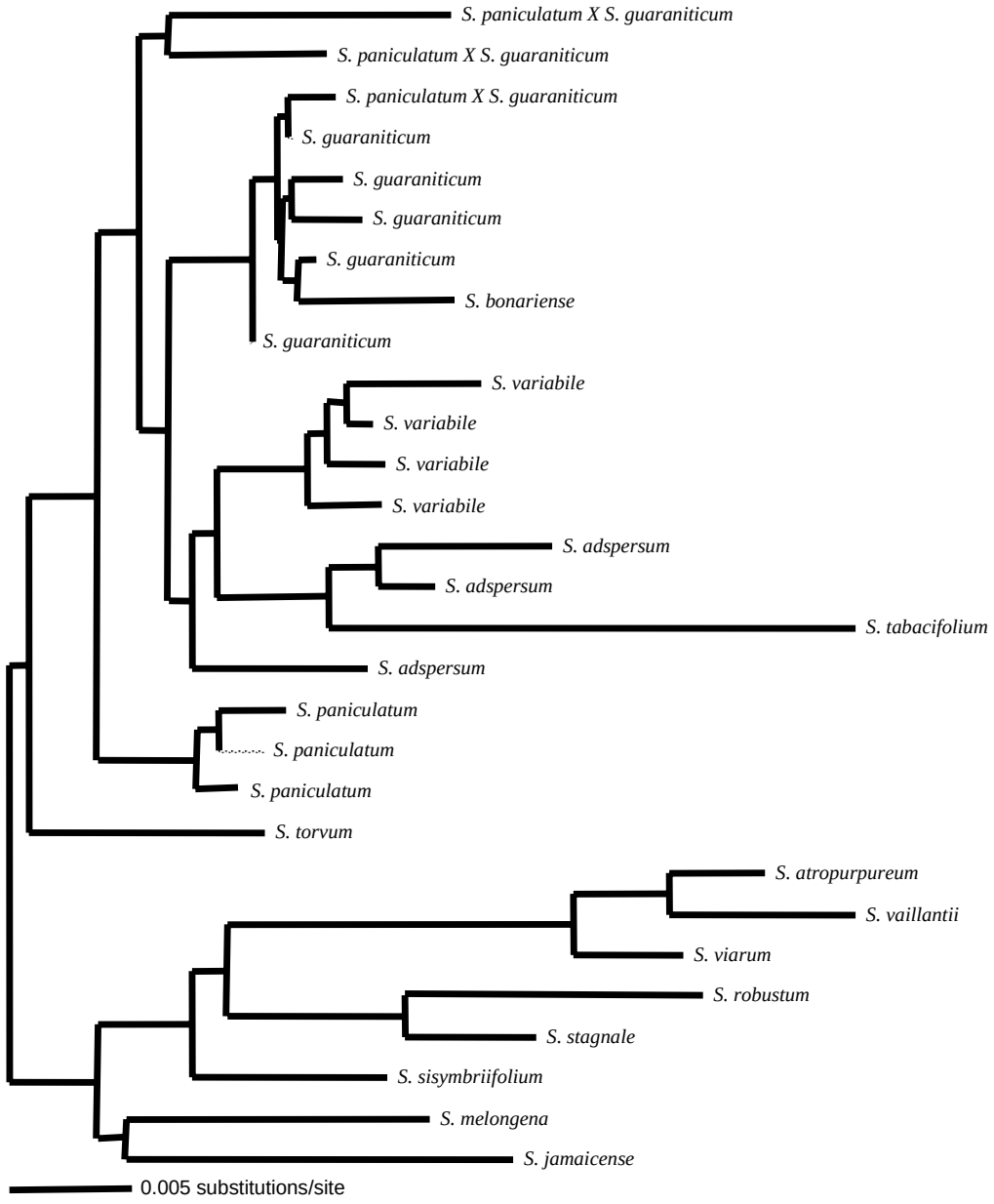
Anexo 24: Árvore filogenética da análise Bayesiana da matriz combinada. (Artigo 2).



Anexo 25: Árvore filogenética da análise de máxima verossimilhança da matriz combinada (Artigo 2).



Anexo 26: Árvore consenso da maioria da análise de parcimônia da matriz combinada, com índels tratados como dados faltantes (Artigo 2).



Anexo 27: Árvore filogenética da análise de distância da matriz combinada (Artigo 2).