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FILOGENIA E DIVERSIDADE GENÉTICA DO GÊNERO *Cunila* D. Royen ex L., (Lamiaceae).

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"... E AI TRANQUEIRA!!!"

(Dr. Sergio)

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ao meu amor Tanara.

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RESUMO

O gênero *Cunila* D. Royen ex L. (Lamiaceae) encontra-se na América de forma disjunta, apresentando dois centros de distribuição de espécies, um na América do Norte e outro na América do Sul o qual apresenta três seções: *Incanae, Incisae* e *Spicatae*. As espécies do gênero *Cunila* são usadas na medicina popular com várias finalidades, o óleo essencial destas espécies apresenta atividades sobre microorganismos patogênicos e insetos. Os resultados obtidos neste trabalho, a partir do seqüenciamento de regiões nucleares e plastidiais, contribuem para elucidar a história evolutiva deste gênero, sendo este o primeiro relato filogenético para o gênero *Cunila*. Baseado nos resultados fortemente apoiados pelos diversos tratamentos estatísticos se pode observar a parafilia do gênero *Cunila,* sugerindo-se ainda a união de duas seções geneticamente muito similares, as seções arbustivas *Incanae* e *Incisae*. Paralelamente a este estudo, os marcadores moleculares ISSR foram aplicados visando contribuir para a classificação das espécies Sul-Americanas. Os resultados obtidos apontam para a separação destas espécies em dois grupos: arbustos e subarbustos, reforçando os resultados referentes à análise filogenética do gênero. O grupo de espécies arbustivas foi formado pelas seções *Incanae* e *Incisae*, enquanto que o grupo subarbustivo foi formado pela seção S*picatae*. Os marcadores moleculares ISSR foram empregados, igualmente, no estudo de variabilidade genética dentro e entre populações de *Cunila menthoides*. Cada população foi caracterizada como um "pool" gênico distinto correspondendo a sua distribuição geográfica, apresentando baixa variabilidade intrapopulacional, e indicando que cada população é derivada de um limitado número de plantas com baixa troca gênica entre as populações. Diferentes populações de *C. menthoides* foram sujeitas à hidrodestilação por

arraste a vapor e seu óleo essencial analisado em GS e GS–MS. Dois diferentes quimiotipos foram observados: pulegona e linalol, sendo que alguns indivíduos apresentaram variações relativas entre as percentagens de pulegona e mentona. Na rota biossintética dos monoterpenos, pulegona pode ser reduzido a mentona, pela ação da enzima pulegona redutase (PR). A alta ou baixa expressão da PR pode resultar no respectivo aumento ou diminuição de produção de mentona ou pulegona. O quimiotipo linalol foi formado unicamente por uma população, enquanto que todas as outras populações estudadas integraram o grupo caracterizado por pulegona. A seção *Incanae* é composta por uma única espécie: *Cunila incana*. A análise de seu óleo essencial demonstrou alta percentagem de sesquiterpenos, não sendo esta uma característica do gênero, sendo que todas as pesquisas realizadas com óleos essenciais de outras espécies do gênero indicaram altas concentrações de monoterpenos, e por conseqüência, poucos compostos sesquiterpênicos.

Os resultados obtidos contribuem para o conhecimento químico e genético deste gênero com enorme potencial aromático e medicinal, o qual possui ampla distribuição no Rio Grande do Sul.

ABSTRACT

The genus *Cunila* D. Royen ex L. (Lamiaceae) is an american genus and presents two centers of distribution, one in North America and another in the southern of South America. It´s species are classified into three sections: *Incanae, Incisae* and *Spicatae*. *Cunila* species are used in folk medicine for a lot purposes, moreover, *Cunila* essential oils have compounds responsible for antibacterial, antifungal and insecticidal activity. The results obtained in this work, based on the sequencing from nuclear and chloroplast sets, contributed to clarify the origin and evolution of these species, being the first phylogenetic report for this genus. Based on the strongly statistical supported results the paraphyly of *Cunila* were reported and we suggest the union of the two South American sections formed by shrubs species (*Incisae* and *Incanae*). In addition, the molecular markers ISSR were applied to contribute with the classification of the South American species. The results showed two main clusters, one consisting of shrubs and the second by subshrubs species, which refforces the data showed in the phylogenetic study. The shrub group was composed by species representing *Incanae* and *Incisae* sections, meanwhile the subshrub group was characterized by the species from S*picatae* section. The molecular markers ISSR were also applied to access inter and intrapopulational genetic variability of *Cunila menthoides*. Each population were characterized as a distinct genetic pool according with the geographic distribution, populations analyzed were genetically structured with low genetic variability, indicating that each population derives from a limited number of plants with low gene flow between populations. Air-dried samples of individual plants of different populations of *C. menthoides* were extracted by steam distillation and analyzed using GS and GS–MS. Two different chemotypes were

observed: pulegone and linalool. Some samples showed a variation in the percentage of menthone/pulegone. In the monoterpenes biosynthetic pathway, pulegone can be reduced to menthone, by pulegone reductase (PR). Overexpression and cosuppression of PR can result in the respective increase or decrease in the production of menthone and pulegone. The linalool chemotype was formed by only one population; meanwhile all the other populations analyzed composed the group characterized by pulegone. The *Incanae* section is represented by *C. incana*. The essential oil obtained from this species showed high concentrations of sesquiterpenes, differing of the essential oil obtained from the other *Cunila* species, which is characterized by high concentration of monoterpenes and consequently low concentrations of sesquiterpenes compounds.

The results obtained in this work, corresponding to a increase in the chemical and genetic knowledge of this genus which represent a great aromatic and medicinal potential, and show large distribution in the Rio Grande do Sul State.

INTRODUÇÃO GERAL.

O gênero *Cunila* D. Royen ex L.

O gênero *Cunila* D. Royen ex L. pertence à família Lamiaceae, subfamília Nepetoideae**,** tribo Mentheae (Cantino, 1992). Muitas espécies deste gênero são conhecidas popularmente pelo nome de "poejo" ("poleo" no Uruguai, Argentina e México), sendo que algumas variações dos nomes populares se relacionam, geralmente, com o habitat (poejo-do-banhado, poejo-do-brejo, poejo-do-campo) e/ou com o tamanho das folhas ou porte da planta (poejo-de-folha-grande, poejo-de-folha-miúda, poejinho) (Bordignon *et al*., 1997).

O gênero *Cunila* ocorre na América de forma disjunta, apresentando dois centros de distribuição, um na América do Norte representado por uma espécie do Leste dos Estados Unidos (*C. origanoides* (L.) Britton), e oito espécies do México, sendo sete do ocidente e centro do país *(C. longiflora* Gray, *C. secunda* S. Watson, *C. crenata* M.R. Garcia-Peña et P. Tenório Lezama, *C. lythrifolia* Benth., *C. polyantha* Benth., *C. pycnantha* B.L. Rob. & Greenm., *C. ramamoorthiana* M.R. Garcia-Peña) e uma espécie *(C. leucantha* Kunth ex Schltdl. et Cham.) distribuída do sul do México ao Panamá (exceto Nicarágua); e outro na América do Sul representado por onze espécies, localizadas no sul do Brasil, norte da Argentina e parte do Uruguai (Garcia-Peña, 1989; Bordignon, 1997).

As espécies Sul-Americanas foram classificadas em três seções por Epling (1936), com base no hábito e tipo de inflorescência: *Incanae, Incisae* e *Spicatae*. A seção *Incanae* possui uma única espécie, *C. incana* Benth. e se caracteriza por apresentar flores

solitárias (ou no máximo duas) nas axilas das folhas, estas densamente pilosas com tricomas ramificados. A seção *Incisae* é representada por duas espécies, *C. incisa* Benth. e *C. angustifolia* Benth., as quais são plantas arbustivas com címulas dispostas nas axilas das folhas e com pedúnculos breves de 2-10 mm de comprimento. Por sua vez, a seção *Spicatae* apresenta o maior número de espécies, sendo elas *C. menthoides* Benth., *C. platyphylla* Epling, *C. menthiformis* Epling, *C. spicata* Benth., *C. galioides* Benth., *C. fasciculata* Benth. e *C. microcephala* Benth.. Os representantes desta seção são plantas subarbustivas ou ervas perenes, com címulas sésseis ou com pedúnculos curtos formando espigas ou capítulos globosos ou ovais. Outras duas espécies podem ser incluídas na seção *Spicatae*, *C. tenuifolia* Epling e *Cunila* sp, porém, algumas divergências relativas a esta classificação são relatadas, sugerindo estudos botânicos complementares para estas espécies (Bordignon, 1997).

O gênero *Cunila* é composto por ervas ou arbustos subfrutescentes, eretos ou escandentes com ramos levemente pubescentes a pubescentes. As folhas são simples, opostas, subsésseis, com arranjo regular ou fasciculado, margem crenada, serrada, inteira a levemente serrada ou em algumas espécies revoluta. As inflorescências são terminais ou axilares, com pedúnculos longos, curtos ou ausentes; flores com pedicelos muito curtos ou subsésseis e cálice tubular nervado e dentado, corola sublabiada e 2 estames; núcula sem células mucilaginosas (Epling, 1936, Bordignon, 1997).

A maior parte das espécies do gênero *Cunila* é utilizada na medicina popular, principalmente para o tratamento de afecções respiratórias, como gripes e tosse, embora para muitas espécies não existam referências escritas de uso popular (Simões *et al*., 1994).

A utilização de *C. menthoides* apresenta potenciais consideráveis para indústrias farmacêuticas, cosméticas e alimentícias, pois análises do óleo essencial demonstraram a existência do composto isomentona como majoritário, o qual possui atividade anticonvulsivante, sedativa, analgésica, preventivo de câncer, entre outras (Bordignon *et al.*, 1998; Duke, 1994). *Cunila incisa*, espécie arbustiva encontrada em beiras de estradas, contém em seu óleo essencial altos teores do composto 1,8-cineol (Bordignon *et al*., 1996; Agostini *et al*., 2006). Este composto apresenta atividades anestésica, antisséptica, antibronquítica, antilaringinítica, antifaringinítica, expectorante, entre outras (Duke, 1994).

Segundo Bordignon *et al*. (1997), a espécie de maior utilização popular é *C. microcephala*, sendo que no Rio Grande do Sul suas folhas são adicionadas ao chimarrão promovendo aroma e sabor, ainda, suas folhas e flores são usadas como estimulante e no tratamento de tosses crônicas e em afecções respiratórias (Simões *et al*., 1994), enquanto que *C. galioides*, espécie de ocorrência mais freqüente no norte do Rio Grande do Sul, é considerada expectorante, laxante e calmante (Lopes *et al*., 1988).

Outras citações sobre o uso popular de espécies do gênero *Cunila* podem ser observadas. *Cunila fasciculata*, espécie de menor distribuição, é citada como aromatizante e para o combate a doenças respiratórias, bem como *C. menthiformis*. *Cunila angustifolia*, representante da seção *Incisae*, por sua vez apresenta o mesmo uso de *C. microcephala*, dando aroma e sabor ao chimarão, bebida típica do Rio Grande do Sul. Por sua vez, *Cunila spicata* é conhecida por seus efeitos béquicos e sudoríparos (Bordignon, 1997). Para as espécies *C. platyphylla* e *C. incana* não foram encontradas referências quanto ao uso medicinal popular.

As espécies Norte-Americanas apresentam igualmente diversos usos populares. Entre as espécies mexicanas, *C. lythrifolia* é empregada no combate a enfermidades respiratórias e gastrointestinais (Hernandez *et al*., 1989), enquanto que *C. polyantha* é empregada no alívio de cólicas e ainda é indicada como tônico sanguíneo (House *et al*., 1995). Para o restante das espécies mexicanas, não foram encontrados usos populares na literatura.

Cunila origanoides, a única espécie nativa nos Estados Unidos da América, conhecida popularmente pelo nome de "American Dittany", é usada em chás com o objetivo de minimizar febres e dores de cabeça, apresenta ação diaforética, emenagoga e estimulante, além de ser considerada antiséptica. Esta espécie pode ainda ser empregada em paisagismo, sendo usada para embelezar jardins, de forma isolada ou junto a rochas ou bordadura de jardins (Duke, 1994).

As espécies do gênero *Cunila* compreendem habitats bastante característicos. As espécies *C. galioides* e *C. spicata* apresentam uma maior área de distribuição, ocorrendo em locais úmidos e abertos. Em contrapartida, *C. fasciculata* é a espécie que apresenta a área de distribuição mais restrita, podendo ser considerada como endêmica do Rio Grande do Sul, encontrando-se na lista de espécies ameaçadas de extinção (http://www.sema.rs.gov.br/sema/html/pdf/especies-ameacadas.pdf).

As espécies da seção *Spicatae*, em sua maioria, habitam locais úmidos, tais como depressões brejosas, orla de banhados, beira de cursos de água e orla de matas com exceção da espécie *C. menthoides*, que como *C. incana* (seção *Incanae*), habita preferencialmente campos secos e muitas vezes pedregosos, localizados em encostas de morros e colinas. As duas representantes da seção *Incisae*, *C. incisa* e *C. angustifolia*, são

arbustos que ocorrem preferencialmente em orla de matas, característica comum de muitas Lamiaceae americanas (Hedge, 1992), e também na beira de estradas.

A espécie de maior uso popular, *C. microcephala*, ocorre preferencialmente em locais úmidos, como o caso de orla de banhados, sendo comum na região da Serra do Sudeste no Rio Grande do Sul e no Uruguai. Esta espécie pode ser encontrada sob cultivo, pois é freqüentemente utilizada como medicinal; tal fato gera dúvidas quanto a sua ocorrência natural em outras regiões, fora do Rio Grande do Sul e Uruguai, pois estas poderiam ser plantas oriundas de escapes de cultivo (Bordignon, 1997).

Segundo Bordignon (1997), espécies como *C. microcephala*, *C. fasciculata* e *C. platyphylla*, podem ser encontradas em densos aglomerados, em áreas onde não há pastoreio, em muitos casos, eliminando outras espécies de plantas que ocorrem nestes locais. Este comportamento pode estar ligado à presença de mentofurano nos óleos voláteis de *C. microcephala* e *C. fasciculata* e de pulegona em *C. platyphylla*, substâncias estas com reconhecida atividade alelopática (Von Poser *et al.*, 1996, Macias *et al.*, 1989).

Apesar do potencial aromático e medicinal das plantas do gênero Cunila, foram registrados até o momento apenas dois trabalhos na área de micropropagação ou cultura de tecidos, sendo estes com C. galioides (Fracaro e Echeverrigaray, 2001) e C. incisa (Agostini e Echeverrigaray, 2006b). Plântulas micropropagadas apresentaram características fenotípicas e fenológicas normais, tornando este sistema eficiente para a produção de mudas uniformes, visando a utilização sustentável desta espécie.

***Cunila menthoides* Benth.**

Dentro do gênero *Cunila*, a espécie *Cunila menthoides*, ainda pouco estudada, apresenta potencial tanto na área médica quanto cosmética, devido à presença de altas concentrações dos monoterpenos isomentona, mentona, pulegona e linalol, conferindo à espécie efeitos anticonvulsivante, sedativo, analgésico, preventivo de câncer, entre outros, quando utilizada como medicinal (Bordignon, 1997).

Cunila menthoides é uma erva ereta ou ascendente fortemente aromatica, de 20 a 30cm de altura. O caule quando jovem é densamente coberto por tricomas simples e cônicos. Apresenta folhas subcoriáceas, ovais, com tricomas simples, cônicos e antrosos, mais longos nas nervuras da face inferior e pecíolos, apresentando ainda glândulas sésseis em ambas as faces, tendo seus pecíolos 0,3 a 10 mm de comprimento. As inflorescências apresentam-se como glomérulos arredondados, interruptos, com pedicelo medindo 0,5 a 0,75 mm de comprimento. O cálice apresenta tubo de 2,5 mm, dentes subiguais, dois maiores, aciculares, ciliados, 2,5 a 3 mm de comprimento e três menores com 1,5 mm de comprimento, aproximadamente. O cálice florífero apresenta tubo com 2 a 2,5 mm, sua corola é lilás, com tubo de 2 a 3,5 mm de comprimento. Os estames são exsertos, com 3 mm, e o estigma tem 5 mm, também exserto. As núculas são oblongas, marrons, com 1 mm de comprimento e 0,5 mm de largura (Bordignon, 1997).

A espécie *C. menthoides*, pertencente à sessão *Spicatae*, é conhecida popularmente pelo nome de poejo-de-folha-grande (Bordignon *et al.*, 1997), é uma espécie nativa Sul-Americana, relatada apenas para o Rio Grande do Sul (Epling, 1936, 1939), porém recentemente coletada em Lages, Santa Catarina (Brasil) e Montevideo

(Uruguai). No Rio Grande do Sul esta espécie é mais freqüente na Serra do Sudeste (Amaral Ferrador e Camaquã), com raras coletas em outras regiões, como Campanha (Alegrete) e Depressão Central (Santa Maria). No norte do Estado do Rio Grande do Sul, recentemente coletou-se esta espécie em Vacaria, próximo ao município de Lages, SC.

Esta espécie, muito aromática, pode ser observada florescendo e frutificando durante os meses da primavera, com início da floração em setembro, estendendo-se até novembro, sendo que seus cálices frutíferos podem ser encontrados até dezembro. Esta espécie apresenta xilopódio, podendo ser encontrada em campos pedregosos na encosta de cerro, formando touceiras (Bordignon *et al.*, 1997; Coelho de Souza, 1997).

Apenas um trabalho referente a *C. menthoides* é encontrado na literatura, o qual trata da descrição dos componentes químicos do seu óleo (Bordignon *et al.*, 1998). Não há trabalhos genéticos nem tão pouco químicos com ênfase populacional acerca desta espécie.

***Cunila incana* Benth.**

Cunila incana Benth. pertence a seção *Incanae*, e é a única representante desta seção. Esta espécie é um arbusto de 1 a 2 m de altura. As folhas são oblanceoladas a espatuladas com 9,5 a 11 mm de comprimento e 3,8 a 4,3 mm de largura, com tricomas ramificados e glândulas sésseis, e margens lisas ou crenada na metade superior. O pecíolo mede cerca de 3 mm de comprimento. As inflorescências são isoladas, com aproximadamente 4 mm de comprimento (cálice e corola), surgindo nas axilas das folhas.

O cálice tem coloração amarelada e é externamente incano-tomentoso com tricomas e glândulas sésseis. A corola é externamente pilosa com tricomas simples e glândulas sésseis. As núculas ovóides, lisas, apresentando cor marrom, com aproximadamente 0,75 mm de comprimento (Bordignon, 1997).

Cunila incana pode ser encontrada no Brasil nos estados do Paraná, Santa Catarina e Rio Grande do Sul, existindo coletas no Uruguai e Argentina (Epling, 1936, 1939). No estado do Rio Grande do Sul esta espécie é encontrada principalmente na Serra do Sudeste e Campanha, e raramente em outros locais, como Depressão Central (Porto Alegre) e Litoral (Torres).

Esta espécie, consideravelmente menos aromática que o restante das representantes do gênero *Cunila*, pode ser observada florescendo e frutificando entre os meses de Setembro e Dezembro, sendo encontrada em campos secos, muitas vezes pedregosos e também em barrancos na beira de estradas (Bordignon *et al.*, 1997; Coelho de Souza, 1997).

Apenas um trabalho referente a *C. incana* é encontrado na literatura, o qual trata da descrição de flavonas e flavononas de algumas espécies de *Cunila* Sul-Americanas. Segundo os autores, apenas flavonas metoxiladas foram isoladas desta espécie (Bordignon *et al*., 2003).

Óleos essenciais do gênero *Cunila*, fonte medicinal e aromática.

As rotas bioquímicas das plantas estão divididas em metabolismo primário e secundário. O metabolismo primário produz substâncias essenciais para a sobrevivência

das plantas, tais como: açúcares, ácidos graxos, ácido mevalônico, aminoácidos, acetil-coenzima A, nucleotídeos e seus polímeros derivados, entre outros (Di Stasi, 1996; Herbert, 1989). No metabolismo secundário, são sintetizadas e acumuladas substâncias responsáveis pela adaptação destas ao meio em que vivem (Bates, 1985). Dentre estas adaptações podemos citar: defesa contra patógenos e predadores, adaptações a mudanças climáticas bruscas, inibição de germinação e crescimento de outros vegetais (substâncias alelopáticas) e atração de agentes polinizadores e de disperção de sementes (Rotgé, 1991).

O metabolismo secundário origina grande variedade de substâncias naturais, as quais podem ser utilizadas por diferentes indústrias: farmacêutica (fármacos: taxol, efedrina), alimentícia (flavorizantes e corantes naturais), cosmética (cânfora, linalol), química, agroquímica (fungicida, inseticida) entre outros (Bates, 1985; Evans, 1991).

Dentre os metabólitos secundários, um dos grupos mais importantes de matérias-primas são os óleos essenciais ou essências. A ISO - International Standard Organization - define óleos essenciais como produtos obtidos de partes de plantas através de destilação por arraste a vapor de água, bem como os produtos obtidos por expressão dos pericarpos de frutos cítricos. Os óleos essenciais apresentam algumas características fisico-químicas consideráveis: são geralmente líquidos, de aparência oleosa à temperatura ambiente, voláteis, solúveis em solventes orgânicos apolares, e geralmente não são muito estáveis na presença de luz, calor, umidade e metais (Shreve e Brink, 1980; Costa, 1994).

Os óleos essenciais podem ter sua composição variada de acordo com a época da colheita, localização geográfica, pelas características genéticas da planta (Costa, 1994), ou mesmo em seus diferentes estágios de desenvolvimento (Bourbott e Loomis, 1967;

Shalaby e Verzar-Petri, 1978). Existem diferenças significativas na composição dos óleos essenciais de uma espécie para outra, além do que, alguns compostos podem se apresentar repetidos em diferentes espécies (Putievsky *et al.*, 1990; Guillén *et al.*, 1996). Diferenças na composição dos óleos essenciais dentro de plantas de uma mesma espécie provindas de regiões geográficas distintas ou da mesma região geográfica têm sido freqüentemente evidenciadas (Guillén, 1996; Echeverrigaray *et al*., 2003).

A diferença observada na composição de óleos essenciais em plantas da mesma espécie é um fato a ser levado em consideração. As diferenças qualitativas encontradas são fundamentalmente de base genética (Hay e Waterman, 1993), enquanto que as variações quantitativas podem ser atribuídas a modificações ambientais (edafológicas ou climáticas) e a fatores genéticos (Grella e Picci, 1988).

A composição química dos óleos essenciais é bastante variada, podendo apresentar hidrocarbonetos, álcoois, aldeídos, cetonas, fenóis, ácidos, éteres, ésteres, lactonas, compostos nitrogenados e compostos sulfurados (Shreve e Brink, 1980).

Um grande número de compostos presentes nos óleos essenciais apresenta atividade biológica de interesse, tais como, ação antiinflamatória, pesticida, inseticida, antiséptica, herbicida, antioxidante, entre outras (Duke, 1994). Entre estes compostos, podemos citar o d-limoneno (anticancerígeno, herbicida, insetífugo e inseticida), o citral (antiestamínico, bactericida, preventivo de câncer e herbicida), o linalol (antisséptico, insetífugo e termitífugo), o timol (antihelmintico, antiinflamatório, antiséptico, bactericida, fungicida, larvicida e vermicida), o 1,8-cineol (alelopático, anestésico, antibronquítico, antisséptico, bactericida, expectorante, herbicida e insetífugo), entre outros.

Os óleos essencias de origem vegetal são importantes também nas indústrias alimentícias, não somente por seu uso como flavorizantes, mas por suas atividades antioxidantes (Guillén, 1996), além do que, podem ser usados na produção de aromas e sabores de alimentos e bebidas (Di Stasi, 1996).

Vários procedimentos podem ser empregados para a extração dos óleos essenciais, levando-se em consideração a natureza da essência a se extrair. Os métodos mais utilizados para a obtenção dos óleos essenciais são: destilação a vapor (hidrodestilação e destilação por arraste a vapor), *enfleurage*, expressão, extração com solventes voláteis e extração com fluído supercrítico (Atti-Serafini *et al*., 2002). A destilação por vapor é o método mais usado geralmente para produção industrial de óleo essencial (Moyler, 1998).

Os óleos essenciais estão presentes na maior parte das espécies vegetais, entretanto, algumas famílias se destacam pelo grande número de espécies com alta produção de óleos de composição e atividade biológica de interesse. As plantas da família Lamiaceae são consideradas fontes de inúmeros compostos químicos biologicamente ativos que lhes conferem atividades terapêuticas (Sur *et al.*, 1991; Costa, 1994; Di Stasi, 1996). Esta família é considerada uma das mais ricas quanto à presença de óleos voláteis. Segundo Lawrence (1992), aproximadamente 40% dos seus 200 gêneros possuem espécies aromáticas, incluindo o gênero *Cunila*. Nesta família encontram-se representantes condimentares, aromáticos e medicinais muito populares, utilizados na forma de chás na medicina popular, na culinária, em indústrias alimentícias, perfumarias e na preparação de medicamentos. Dentre estes podemos citar: lavanda (*Lavandula* L.), sálvia (*Salvia officinalis* L.), tomilho (*Thymus vulgaris* L.), alecrim (*Rosmarinus*

officinalis L.*)*, manjericão (*Ocimum basilicum* L.), orégano (*Origanum vulgare* L.), manjerona (*Origanum majorana* L.), menta (*Mentha* L.), entre outros.

Ainda pouco estudadas, as espécies do gênero *Cunila* são usadas na medicina popular como estimulantes, antiespasmódicas, emenagogas, antifebris, no tratamento de tosses crônicas e infecções respiratórias (Simões *et al.*, 1994) e ainda como aromatizantes da bebida tradicional do Rio Grande do Sul, o chimarrão (Bordignon, 1997). Além disso, o óleo essencial de espécies de *Cunila* apresenta atividade bactericida, fungicida e inseticida (Duke, 1994; Luz *et al.*, 2006), sendo testado contra microrganismos de alimentos (Sandri *et al.*, 2007), bem como no controle de insetos e proliferação de bactérias patogênicas em aviários (Prado, 2007).

A composição química do óleo essencial das plantas deste gênero apresenta grande variação. Bordignon (1997) avaliou a composição química do óleo essencial de seis espécies de *Cunila* baseado em espectrometria GC e GC-Massa e espectroscopia 13C-NMR. O rendimento de óleo v/p variou entre 0,16% para *C. menthiformis* e 1,30% para *C. incisa*. Para as espécies avaliadas foram encontrados os seguintes componentes majoritários: pulegona em *C. platyphylla* (71,2 %); isomentona em *C. menthoides* (88.8 %); 1,8-cineol em *C. incisa* (>50%); óxido trans-piperitona (42 %) e sabineno (41 %) em *C. angustifolia* e por fim, o principal constituinte tanto em *C. microcephala* quanto em *C. fasciculata*, é o mentofurano, 82,3% a 85,1% e 71,6% a 76,4%, respectivamente.

Estudos fitoquímicos realizados com *C. spicata* por Manns e Hartmann (1992), revelaram altas concentrações de betulenal, isorosiridol, hidroperoximonoterpenos, sitosterol, fitol, cadinol, linalol e geraniol. O óleo essencial desta espécie apresenta comprovado efeito protetor contra convulsões crônicas (Coelho de Souza, 1997).

Moreira e Krambeck (1976), por sua vez, observaram altas concentrações de pulegona (49,6%), mentona (38,7%) e mentol (4,3%) em *C. angustifolia*. Os dados obtidos por estes autores, comparados aos dados obtidos por Bordignon (1997) demonstram a existência de quimiotipos dentro desta espécie. A existência de diferentes compostos majoritários dentro de uma mesma espécie também foi observada no estudo populacional para *C. galioides*, realizado por Echeverrigaray *et al*. (2003). O primeiro quimiotipo identificado apresentou alta concentração de citral, com duas formas isoméricas: neral e geranial. O segundo com alta concentração de ocimeno e o último com alta concentração de derivados de mentona. Seguindo esta linha de análises Echeverrigaray *et al*. (2008), relataram quatro quimiotipos para *C. spicata*: linalol/1,8-cineol, 1,8-cineol, carvona/carveol, e 1,8-cineol/limoneno.

Outro estudo comparando a composição do óleo de diferentes populações da mesma espécie foi realizado. Uma ampla gama de substâncias foi identificada no óleo de *C. incisa*, sendo o composto majoritário o 1,8-cineol (40-60%), sendo que este dado confirma os dados prévios obtidos por Bordignon (1997). Neste trabalho não foram observados quimiotipos entre as diferentes populações (Agostini *et al*., 2006). O fato de uma planta apresentar um composto na concentração acima citada, ou mesmo mais alta, permite a obtenção de um óleo essencial de alta pureza.

Para a espécie mexicana *C. lythrifolia*, Manjarrez e Mendonza (1966) obtiveram os seguintes compostos majoritários: β-ionona (24.3%), óxido de linalol (27.6%), linalol (13.0%) e acetato de citronelila (11.0%). Cicció e Poveda (1999), por sua vez, observaram os seguintes compostos majoritários para *C. polyantha*: mentona (63%) e

pulegona (14%). Para o restante das espécies mexicanas não foram encontrados dados referentes à composição química do óleo essencial.

Cunila origanoides, nativa da América do Norte, apresentou os seguintes compostos majoritários: thimol (37,7%) e gamma-terpineno (27,0%) (Lawrence, 1989).

Os dados anteriormente citados mostram a grande variabilidade existente quanto à composição dos óleos essenciais dentro das espécies do gênero *Cunila*, e o potencial delas como plantas aromáticas e medicinais de valor econômico.

Marcadores moleculares e sua utilização em plantas aromáticas e medicinais.

Marcadores moleculares evidenciam características do DNA que diferem dois ou mais indivíduos e são herdadas geneticamente. Segundo Ferreira e Gratapaglia (1996), marcadores moleculares apresentam algumas vantagens sobre os marcadores morfológicos. Alguns marcadores moleculares apresentam-se como co-dominantes, ao passo que marcadores morfológicos são, em sua maioria, dominantes ou recessivos (Tanksley, 1983; Beckmann e Soller, 1988; Burr *et al*., 1983; Stuber, 1992). Os marcadores moleculares podem ser empregados para caracterizar o genótipo de um indivíduo em qualquer estágio de desenvolvimento a partir de amostras de tecidos, ou mesmo de células, apresentando vantagem em termo de tempo, além do que não são afetados por fatores ambientais, permitindo assim a determinação de relações filogenéticas entre materiais de origens distintas (Swofford *et al*., 1996), entre outras.

Os diversos tipos de marcadores moleculares disponíveis diferenciam-se pela tecnologia utilizada, pela habilidade de detectar diferenças entre indivíduos, custo,

facilidade de uso, consistência e repetibilidade. Segundo Milach (1998), a metodologia utilizada para identificar os tipos de marcadores moleculares divide-os em dois grupos: hibridização ou amplificação de DNA.

Entre os marcadores mais conhecidos identificados por hibridização estão os marcadores RFLP (Restriction Fragment Length Polymorphism; Botstein *et al*., 1980) e minissatélites ou locos VNTR (Variable Number of Tandem Repeats; Jeffreys *et al*., 1985). Entre os marcadores mais comuns, revelados por amplificação, temos: RAPD (Random Amplified Polymorphic DNA; Williams *et al*., 1990); ISSR (Inter Simple Sequence Repeats; Zietkiewicz *et al*., 1994); SSR ou microssatélites (Simple Sequence Repeat; Litt e Lutty, 1989) e AFLP (Amplified Fragment Length Polymorphism; Vos *et al*., 1995).

São muitos os aspectos considerados na escolha do tipo de marcador a ser utilizado, não existindo, assim, aquele que possa ser considerado superior para todos os atributos (Milach, 1998).

Um dos fatores mais limitantes destes métodos é a baixa reprodutibilidade do RAPD, o alto custo do AFLP e a necessidade de conhecimento prévio das seqüências alvo para desenvolver “primers” espécie-específicos para acessar polimorfismos baseados em microssatélites (Reddy *et al*., 2002).

ISSR-PCR é uma técnica que se sobrepõe a estas limitações (Zietkiewicz *et al.*, 1994; Gupta *et al*., 1994; Wu *et al*., 1994; Meyer *et al*., 1993). Neste método SSRs são usados como “primers” para amplificar as regiões entre seqüências SSRs, sendo estas curtas repetições em tandem (STRs) ou repetições em tandem de número variável (VNTRs) de 1 a 4 bases de DNA, presentes em genomas eucariotos (Tautz e Renz, 1984).

A técnica de ISSR é baseada em PCR, envolvendo a amplificação de um segmento de DNA presente entre duas seqüências idênticas de microssatélites orientadas em direções opostas. ISSR utiliza microssatélites, normalmente 16 a 25 bp de comprimento, como "primer" em uma reação de PCR, visando amplificar regiões inter-SSR de diferentes tamanhos. Os "primers" podem envolver repetições di-, tri-, tetra- ou penta-nucleotídicas, ainda podendo ser não-ancorados (Gupta *et al.*, 1994; Wu *et al*., 1994; Meyer *et al*., 1993) ou ancorados na região 3´ou 5´ com 1 a 4 bases degeneradas (Zietkiewicz *et al.*, 1994). ISSR combina muitos benefícios das análises de AFLP e microssatélites com a universalidade do RAPD. Marcadores ISSR apresentam alta reprodutibilidade possivelmente devido ao uso de "primers" longos (16 a 25 pb) quando comparado com os "primers" do marcador RAPD (10 pb), permitindo o uso de altas temperaturas de anelamento (45 a 60°C), o que conduz à alta adstringência. A temperatura de anelamento depende da quantidade de GC presente no "primer" utilizado e normalmente varia entre 45 a 65°C (Reddy *et al*., 2002).

Segundo Reddy *et al*. (2002), quando "primers" ancorados na região 5´ são utilizados, os produtos de amplificação incluem a seqüência de microssatélite e suas variações de tamanho no genoma e assim fornece maior número de bandas e maior grau de polimorfismo. Usualmente repetições di-nucleotídicas, ancoradas na região 3´ou 5´revelam alto polimorfismo (Blair *et al*., 1999; Joshi *et al*., 2000; Nagaoka e Ogihara, 1997).

A eficiência desta técnica foi evidenciada por vários trabalhos (Ajibade *et al*., 2000; Adams *et al*., 2003; Sudupak, 2004; Wu *et al*., 2004; Shi *et al*., 2006; Zhao *et al*., 2006), sendo que alguns destes utilizaram pequeno número de "primers": Charters *et al.*

(1996) utilizaram três “primers” ancorados na região 5` para distinguir entre 20 cultivares de *Brassica napus* L.. Em um estudo com *Lupinus albus* L. observou-se que entre 10 “primers” utilizados, dois já eram suficientes para distinguir todos os 37 acessos estudados (Gilbert *et al.*, 1999). Similar a este trabalho, quatro “primers” foram suficientes para distinguir 34 cultivares de batata (Prevost e Wilkinson, 1999) e três “primers” puderam distinguir 16 genótipos de *Ribes* L. (Lanham e Brennan, 1998). O uso de poucos “primers”, porém altamente informativos baixam o custo, o tempo e o labor das análises de diversidade (Reddy *et al*., 2002).

ISSR-PCR é uma técnica simples, rápida e eficiente, além de ser de alta reprodutibilidade. O uso de material radioativo não é necessário bem como não existe necessidade de conhecimento prévio das seqüências a serem amplificadas. Ainda, diferentes combinações de “primers”, âncoras e comprimento de “primers” podem ser utilizados visando diferentes formas de acessar a variabilidade. Os “primers” são longos, conduzindo à alta adstringência. Os produtos amplificados são usualmente de 200 a 2000bp e podem ser detectados tanto em gel de poliacrilamida quanto em gel de agarose (Reddy *et al*., 2002).

Marcadores moleculares têm sido utilizados com sucesso em estudos de plantas aromáticas e medicinais, principalmente na determinação de diversidade genética, caracterização genética de quimiotipos, identificação de materiais comerciais, e determinação de pureza varietal (Skoula *et al*., 1999; Vazquez *et al*., 1999; Echeverrigaray *et al*., 2001; Kimball *et al*., 2001; Vieira *et al.*, 2001; Matter *et al*., 2001; Agostini, 2003; Albuquerque, 2004; Fracaro *et al*., 2005; Agostini e Echeverrigaray, 2006; Fracaro e Echeverrigaray, 2006; Kochieva *et al*., 2006; Liu *et al*., 2006). Apesar da

eficiência destes marcadores, o número de trabalhos publicados em plantas aromáticas e medicinais até o presente é ainda insipiente.

Apesar da larga utilização popular, e do potencial das espécies do gênero *Cunila* como plantas aromáticas e medicinais, há hoje, pouca referência quanto a estudos fitogeográficos, ecológicos, ou genéticos das mesmas. Os únicos trabalhos referentes à análise de variabilidade genética dentro do gênero *Cunila* envolvem as espécies *C. galioides* (Fracaro *et al.*, 2005) e *C. incisa* (Agostini, 2003), ambos realizados com marcadores RAPD, e mais recentemente *C. spicata* (Albuquerque, 2004), sendo este o primeiro reportado utilizando-se marcadores ISSR.

Estudo filogenético do gênero *Cunila* D. Royen ex L.

Estudos filogenéticos têm sido realizados utilizando-se diferentes tipos de caracteres, sobretudo morfológicos e moleculares. Atualmente as análises de macromoléculas, em especial o DNA, vem sendo amplamente utilizadas para esse fim, caracterizando uma linha de pesquisa denominada sistemática filogenética molecular. O DNA constitui diretamente o material hereditário, gerando dados de forma rápida e em grande quantidade, graças aos avanços tecnológicos da biologia molecular (Judd *et al.*, 1999; Matioli e Passos-Buenos, 2001; APG II, 2003). Paralelamente a estes avanços, métodos estatísticos de inferência filogenética têm sido elaborados para a obtenção de filogenias robustas, sendo tais ferramentas fundamentais como fonte de informação biológica em diversas áreas (Russo, 2001).

No caso de espécies vegetais, a inferência filogenética pode ser obtida a partir dos três genomas: nuclear, mitocondrial (mtDNA) e plastidial (cpDNA), cada um com características e evolução distintas capazes de resolver questões evolutivas em diferentes níveis taxonômicos (Walbot e Cullis, 1985; Nahum, 2001). O mtDNA e o cpDNA diferem grandemente em relação ao genoma nuclear, em tamanho e número de genes e principalmente nas taxas e padrões de evolução (Nahum, 2001). Segundo Birk (2001), as mitocôndrias e os cloroplastos são herdados de uma maneira não-Mendeliana em todos os organismos estudados, sendo a herança dos genomas citoplasmáticos, freqüentemente materna.

Dentre os genes nucleares, o DNA ribossomal (rDNA) é um dos mais utilizados nos estudos filogenéticos de plantas (El Qualidi *et al*., 1999; Baker *et al*., 2000; Crisp *et al*., 2000; Mackinder, 2000; Prather *et al*., 2002; Murphy, 2003; Mcmahon e Hufford, 2004; Gonzáles e Morton, 2004; Rosselló *et al*., 2006; Walker e Sytsma, 2007). O rDNA constitui uma região do genoma que codifica os componentes do RNA dos ribossomos. O rDNA eucariótico está organizado em tandem com milhares de cópias no genoma. Cada unidade de repetição é formada por genes que codificam a subunidade menor (18S) e a subunidade maior (26S), ambas separadas pelo rDNA nuclear 5,8S. Essas regiões gênicas são separadas pelos espaçadores transcritos internos (ITS), transcritos externos (ETS), assim como, pelos espaçadores não transcritos (NTS) e espaçadores intergênicos (IGS) (Judd *et al*., 1999).

O genoma do cloroplasto corresponde a uma molécula de DNA circular, podendo variar de 120 a 220 kb (Nahum, 2001). O DNA plastidial geralmente apresenta duas regiões simples, uma grande (LSC, large single copy) e uma pequena (SSC, small single

copy), com aproximadamente 134-160 kb e 80-87 kb, respectivamente. Adicionalmente, no cpDNA encontramos regiões repetidas invertidas (IR, inverted repeat), formada por dois segmentos idênticos em sentidos opostos, separando os SSC e LSC (Nahum, 2001). As regiões IR podem apresentar um tamanho variável, de 12 a 25 kb cada, porém algumas linhagens, tais como de algumas leguminosas, têm perdido estas regiões (Palmer e Delwiche, 1998).

Taberlet *et al.* (1991) publicaram "primers" universais para regiões não-codificadoras plastidiais. Dentre as regiões plastidiais não codificadoras mais utilizadas em análises filogenéticas, encontram-se o espaçador intergênico *trn*L-*trn*F e o íntron do gene *trn*L (Mes *et al*., 2000; Holt *et al.*, 2004; Lledó *et al*., 2005). Essas regiões têm sido amplamente utilizadas nas análises filogenéticas nos mais variados níveis taxonômicos (Mes *et al*., 2000; Bruneau *et al.*, 2001; Muschner *et al.*, 2003; Bunsawat *et al*., 2004; Haston *et al*., 2005; Walker e Sytsma, 2007).

Entretanto, o uso de regiões não-codificantes é muito discutido por alguns autores. Segundo Golenberg *et al*. (1993), algumas seqüências não-codificantes contêm mais "indels" (inserções ou deleções) do que substituições, não devendo ser tratados como caracteres informativos. Kelchner (2000) considera que os "indels" podem dificultar o alinhamento das seqüências e a determinação das homologias, mas alega que esses trechos contêm informações filogenéticas importantes e que devem ser incluídas nas análises.

A mitocôndria vegetal, assim como o cloroplasto, apresenta seu material genético na forma de DNA circular. Contudo, o tamanho do seu genoma pode alcançar grandes valores, variando de 6 kb a 2000kb (Judd *et al*., 1999; Nahum, 2001). Pode-se destacar

que o genoma mitocondrial (mtDNA) apresenta um alto dinamismo em algumas espécies com variações no tamanho, porém, seu material genético é muito conservado (Eguiarte *et al*., 2003). Adicionalmente, Muse (2000) verificou que as taxas de substituição são acentuadamente baixas em genes mitocondriais das plantas. Segundo Palmer (1991), o mtDNA de plantas tem sido pouco aplicado em estudos filogenéticos por sua estrutura e taxas de mutação desse genoma. Entretanto, nos últimos anos tem crescido o interesse na inclusão do genoma mitocondrial nos estudos filogenéticos dependendo do nível taxonômico estudado. Alguns trabalhos abrangendo regiões intrônicas e espaçadoras têm mostrado a eficiência dessas seqüências para a sistemática molecular (Freudenstein e Chase, 2001; Duminil *et al*., 2002; Dombrovska e Qiu, 2004)

Apesar da família Lamiaceae apresentar diversos gêneros de plantas aromáticas e medicinais de interesse econômico, incluindo o gênero *Cunila*, poucos trabalhos envolvendo análise filogenética podem ser encontrados na literatura. O espaçador transcrito interno (ITS) do rDNA e a região plastidial *trn*L-L-F têm sido usados com sucesso para avaliar relações ao nível de espécies e de gêneros na família Lamiaceae, conforme demonstrado no estudo filogenético de *Clerodendrum* L. (Steane *et al*., 1999), *Teucrium* L. seção *Polium* (El Qualidi *et al*., 1999), *Stachys* L. (Lindqvist e Albert, 2002); *Monarda* L. (Prather *et al*., 2002), *Mentha* L. (Bunsawat *et al*., 2004), *Conradina* A. Gray (Edwards *et al*., 2006), *Micromeria* Benth. (Meimberg *et al*., 2006), *Rosmarinus* L. (Rosselló *et al*., 2006), *Salvia* L. (Walker *et al*., 2004; Walker e Sytsma, 2007), e *Dicerandra* Benth. (Oliveira *et al*., 2007). Em alguns casos, ambas as regiões foram usadas. Para o gênero *Cunila* não existem relatos de estudos filogenéticos, os quais

podem contribuir para elucidar a história evolutiva e origem dos dois centros de dispersão.

Objetivos

Levando-se em consideração os aspectos medicinais e aromáticos e a utilização popular de diversas espécies do gênero *Cunila*, este trabalho teve como objetivo o estudo filogenético do gênero, e o estudo da variabilidade genética das espécies Sul-Americanas visando-se o conhecimento das relações genéticas entre as espécies, bem como o posicionamento das espécies nas respectivas seções botânicas, definidas até então sem o auxílio de dados moleculares. Também foi objetivo deste trabalho o estudo da variabilidade química e genética da espécie Sul-Americana *C. menthoides*, a qual possui considerável potencial de uso em indústrias farmacêuticas, cosméticas e alimentícias. E finalmente, este trabalho teve também como objetivo, a descrição do conteúdo químico do óleo essencial de *C. incana*. O conhecimento da composição química desta espécie se torna de grande interesse para posteriores trabalhos populacionais visando o conhecimento da variabilidade química existente em seu óleo e assim, a sua utilização com fins econômicos.

CAPÍTULO I

Filogenia do gênero *Cunila* D. Royen ex L.

Phylogenetic inference of the genus *Cunila* D. Royen ex L. based on ITS rDNA and *trn*L-L-F.

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Abstract: *Cunila* species are aromatic and medicinal plants commonly used in folk medicine. This genus presents two centers of distribution, one in North America and another in the southern of South America; the latter being classified into three sections: *Incanae, Incisae* and *Spicatae*. This work uses a phylogenetic approach to study the genetic relationship between South and North American species and among the South American sections. The results obtained in this work, based on the sequencing from nuclear and chloroplast sets, contributed to clarify the origin and evolution of the two centers of distribution, being the first phylogenetic report about this genus. Based on the strongly statistical supported results the paraphyly of *Cunila* were reported. We suggest in this phylogenetic study the union of the two South American sections formed by shrubs species (*Incisae* and *Incanae*), but, despite the efficiency of the methods described here, it is necessary other approaches to confirm this hypothesis.

Key words: Lamiaceae; *Cunila*; *Cunila* sections; phylogeny; Mentheae.

Introduction

The genus *Cunila* D. Royen ex L. belongs to the Lamiaceae family, subfamily Nepetoideae, and tribe Mentheae (Cantino et al. 1992). This genus presents two centers of distribution, one in North America and Mexico formed by nine species and another in the southern of South America with eleven species (Garcia-Peña, 1989; Bordignon, 1997).

The first one is represented by one species from west of the United States (*C. origanoides* (L.) Britton) and eight species from Mexico, being seven from the occident and center *(C. longiflora* Gray, *C. secunda* S. Watson, *C. crenata* M.R. Garcia-Peña & P. Tenorio Lezama, *C. lythrifolia* Benth., *C. polyantha* Benth., *C. pycnantha* B.L. Rob. & Greenm. and *C. ramamoorthiana* M.R. Garcia-Peña) and one species *(C. leucantha* Kunth ex Schltdl. et Cham.) distributed from southern Mexico to Panama (except Nicaragua). The species belonging to the second center were classified into three sections based on growth habit and inflorescence morphology: *Incanae*, *Incisae* and *Spicatae* (Epling, 1936). The section *Incanae* has only one species, *C. incana* Benth. characterized by shrub plants with single hairy pale yellow flowers at upper axils. The section *Incisae* is represented by two species, *C. incisa* Benth. and *C. angustifolia* Benth., characterized by shrubs with inflorescences formed by small glabrous white flowers. The third section, *Spicatae*, is the most important in number of species, formed by eight species: *C. menthoides* Benth., *C. platyphylla* Epling, *C. menthiformis* Epling, *C. spicata* Benth., *C. galioides* Benth., *C. fasciculata* Benth., *C. microcephala* Benth. and *C. tenuiflora* Epling. This section is characterized by subshrubs or evergreen perennial herbs with terminal spikes or globular inflorescences formed by small white almost glabrous flowers.

Both North and Southern species of *Cunila* presents a large variations in their essential oil composition (Manjarrez e Mendoza, 1966; Moreira e Krambeck, 1976; Bordignon et al., 1996, 1997, 1998, 1999; Cicció e Poveda, 1999; Echeverrigaray et al., 2003; Agostini et al., 2006; Echeverrigaray et al., 2008) and are used in popular medicine as stimulants, aromatics, febrifuge, diaphoretic, antispasmodics, emmenagogues, antithermics, or in the treatment of chronic coughs and respiratory infections (Simões et al., 1994), to treat headaches, colds and fevers and in some cases it is believed to induce menstruation and perspiration (Coffey, 1993). Moreover, *Cunila* essential oils have compounds responsible for antibacterial, antifungal and insecticidal activity (Duke, 1994, Sandri et al., 2006).

Despite of their economic potential, species of *Cunila* have been poorly studied genetically within Lamiaceae (Fracaro et al., 2005; Agostini, 2003 and Albuquerque, 2004). The taxonomy of the genus, particularly in the case of South American species, is restricted to morphological analyses made by Epling in the middle of the last century (Epling, 1936, 1951), and to chemotaxonomic data (Bordignon et al. 1996, 1997, 1998, 1999, 2003). More recently a study based on molecular markers ISSR (Inter Simple Sequence Repeat), evolving South American species of *Cunila* confirm the existence of two genetic sections, enclosing the shrubs species *C. incana*, *C. incisa* and *C. angustifolia* in the same section (Agostini et al., 2008).

This work represents the first phylogenetic study using molecular data to the genus *Cunila*. The nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast *trn*L-*trn*L-*trn*F region has been useful for evaluation for generic and species level relationships in the Lamiaceae, as shown by the phylogenetic analysis of *Mentha* L.

(Bunsawat et al., 2004), *Teucrium* L. section *Polium* (El Qualid et al., 1999), *Clerodendrum* L. (Steane et al., 1999), *Monarda* L. (Prather et al., 2002), *Salvia* L. (Walker et al., 2004; Walker and Sytsma, 2007), *Micromeria* Benth. (Meimberg et al., 2006), *Conradina* A. Gray (Edwards et al., 2006), *Rosmarinus* L. (Rosselló et al., 2006) and *Dicerandra* Benth. (Oliveira et al., 2007). Just in some cases both regions were used.

Nuclear ITS and chloroplast *trn*L-*trn*L-*trn*F sequences are certainly popular and frequently the first choice of a great number of researches, as cited above. Chloroplast t*rn*L-*trn*L-*trn*F is undoubtedly easy to amplify and to sequence, including partially degraded DNA from herbarium samples, and became especially popular due to the existence of universal primers described by Taberlet et al. (1991). Nuclear ITS is more contradict, but is one of the best options for nuclear genome and covers the majority of phylogenetic studies (Álvarez and Wendel, 2003). They are relatively easy to amplify, and evolve by a process of concerted evolution, which permits the sequences amplified being treated as a single one. Another reason why ITS is believed to be useful is the high number of nucleotide substitutions because the sequences of spacer regions are non-coding and, in general, evolve more rapidly than coding regions, being attractive to low-level taxonomic studies, like phylogenetic studies at genera level including closely related species (Buckler et al., 1997; El Qualidi et al., 1999; Torrecilla and Catalán, 2002; Guo et al., 2002; Essi and Souza-Chies, 2007; Miz et al., 2008). The disadvantages are linked to its multi-copy nature: the concerted evolution sometimes is not totally efficient, leaving some partially homogenized copies, allowing a certain level of lineage sorting, creating a risk to compare paralogous copies, and the high number of substitutions accumulated can generate homoplasious data and phylogenetic noise.

To test the hypothesis of generic relationships within *Cunila*, three molecular dataset were used: nuclear ITS region, chloroplast *trn*L-*trn*L-*trn*F region and combined matrix (nuclear x chloroplast). Considering the potential of this genus as aromatic and medicinal plants, the aim of the present work was to study the phylogenetic relationship among the South and North American species of *Cunila* to contribute to the knowledge of this genus.

Material and methods

This study encompasses a total of 14 species of the genus *Cunila*, being included all species belonging to the South America, three species from Mexico and the only one native species from Unite States of America (Table 1).

Samples from each species of South American *Cunila* (except for *C. fasciculata*) were collected at different locations in Rio Grande do Sul State between September 2005 and April 2006. *Cunila fasciculata* and *C. tenuiflora*, representative of the *Spicatae* section, were not found during the expeditions. *Cunila fasciculata* is included in the official red list of endanger species of Rio Grande do Sul State (http://www.sema.rs.gov.br/sema/html/pdf/especies-ameacadas.pdf). In this case, we used a dried sample from the herbarium ICN (ICN 35910).

One herbarium sample of the North American species *C. origanoides* (MIN 67984) and the Mexican species C. *lythrifolia* (MIN 13989), *C. polyantha* (MIN 6089) and *C. pycnantha* (MIN 1905) were included to estimate the phylogenetic relationship among North and South American species (Table 1). Because of technical problems during

laboratory work, we could not obtain clearly sequences for *C. pycnantha* for *trn*L-*trn*L-*trn*F.

Rosmarinus officinalis L., belonging to family Lamiaceae and tribe Mentheae were included as outgroup. To test the monophyly of the genus *Cunila*, the following Lamiaceae representatives were used: *Agastache urticifolia* (Benth.) Kuntze, *Agastache rugosa* (Fisch. & C.A. Mey.) Kuntze, *Agastache foeniculum* Kuntze, *Agastache pallidiflora* (A. Heller) Rydb., *Lepechinia chamaedryoides* Epl., *Lepechinia calycina* Epl. ex Munz, *Lepechinia conferta* (Benth.) Epl., *Lepechinia lancifolia* (Rusby) Epl., *Hyptis emoryi* Torr., *Glechon marifolia* Benth., *Glechon thymoides* Spreng., *Hesperozygis spathulata* Epl., *Hedeoma costata* A. Gray, *Hedeoma apiculata* W.S. Stewart. Species, vouchers, locality and GenBank accessions numbers are given in Table 1.

The South American native plants used in this work were determined by Dr. Sérgio A. de L. Bordignon and one specimen for each species was deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (Brazil).

Leaves of silica gel dried or herbarium vouchers were collected for this molecular survey. The native South American samples of *Cunila* were dried in silica gel, except for the herbarium voucher of *C. fasciculata*. Total genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) protocol adapted for minipreparations (Doyle and Doyle, 1987); the DNA extracted from herbarium vouchers of *C. fasciculata* and North American samples were based in the same method but with some changing: addition of 3% β-mercaptoethanol; pellet immerse in solution of 80% ethanol overnight at -20°C to a first wash and 1h at the same temperature to the second wash; DNA precipitation with isopropanol 1h at -20°C; and pellet dry under -4°C overnight.

Amplification of the ribosomal ITS region (ITS1 - 5.8S - ITS2) was performed with one initial 5 minutes step of denaturation at 94°C followed by 40 PCR cycles of 92°C (1min), 58°C (1min) and 72°C (2min) the reactions were completed by a final extension step of 5 min (72°C) and 5 min (10°C), using the two couples of primers (92 + 74 and ITS3 + 75), described by Desfeux and Lejeune (1996). The reactions were prepared in a final volume of 25ul, following standard proportions of reagents, with the addition of 1µl of DMSO (2%) and 50ng of DNA. PCR amplification of the *trn*L–*trn*L-*trn*F region was carried out with one initial 5 minutes step at 94°C followed by 40 PCR cycles of 92°C (1min), 55°C (1min) and 72°C (2min), the reactions were completed by a final extension step of 5 min (72°C) and 5 min (10°C), using the primers C + D and E + F described by Taberlet et al. (1991). A total volume of 25ul standard mixes were prepared, adding 50ng of DNA.

Volumes with 5µl of PCR products were pre-treated with 3.3U SAP (Shrimp Alkaline Phosphatase) and 0.66U of Exonuclease I. The purified products were sequenced directly, in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100, with the same primers used to amplification.

Sequences were previous aligned using the ClustalX 1.81 program (Thompson et al., 1994, 2001) and manually revised using BioEdit 7.0.9 program (Tom Hall, North Caroline State University, EUA). Phylogenetic analyses were performed in PAUP* version 4.0b10 (Swofford, 2000) to Macintosh, Bayesian analysis in MRBAYES version 3.1.2 (Huelsenbeck and Ronquist, 2001) and Mega 4.0 (Tamura et al. 2007) to Windows.

The g1 statistic (Hillis, 1991) was calculated from 10.000 random trees, to measure the phylogenetic information content of the two spacer regions independently and for the combined data.

In order to test the significance of the incongruence between the phylogenetic signals of the DNA fragments, the Partition Homogeneity Test (PHT; Farris et al., 1995), implemented by the software PAUP* 4.0b10 (Swofford, 2000), was carried out.

Considering the result of the PHT performed, the phylogenetic analysis were performed on three datasets (nuclear dataset, including ITS sequences, chloroplast dataset including *trn*L-*trn*L-*trn*F sequences and a combined dataset of both genomic evidences) on PAUP* and Mega, based on Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor-Joining (NJ), and with MRBAYES, based on Bayesian inference (BI).

Maximum Parsimony analysis was conducted on each data matrix, nuclear, chloroplast and combined matrix, without weighting. Each data set was subjected to heuristic searches with tree bisection-reconnection (TBR) as swapping algorithm, MULPARS option, character-state optimization Accelerated Transformation (ACCTRAN) and trees rooted with the outgroup (*R. officinalis*). The addition sequences as-is and random were selected to heuristics searches. These options were applied to the three datasets. All parsimonious trees found from every search were used to compute strict consensus (data not shown) and 50% majority-rule trees. Branch support for the optimal trees found under the parsimony criterion was estimated through 1,000 bootstrap (Felsenstein, 1985) and jackknife (Hedges, 1992) replicates using the full heuristic search options. The level of homologous data in the trees was estimated through Consistency

Index (CI), Retention Index (RI) and Rescaled Consistency Index (RC), implemented by PAUP*.

To analyze the phylogenetic relationships among the ten South American species, Maximum parsimony Exhaustive Search (data not shown) was carried out with the same parameters used in parsimony.

The Bayesian inference and likelihood searches were performed with the appropriate model of nucleotide substitution determined using the MODELTEST 3.06 program (Posada and Crandall, 1998). This procedure implements a hierarchical likelihood-ratio test to determine the model that best fits the data.

Likelihood analyses were performed with heuristic searches with random-addition and addition of taxa type as-is, TBR branch swapping, and MULPARS option. The models applied were: GTR+I+G for all datasets. Branch support for the optimal trees found under the likelihood criterion was estimated through 1,000 bootstrap and jackknife replicates using full heuristic search options. Outgroup was the same as above described for MP.

Bayesian inference used the same substitution models for three data matrixes (GTR+I+G) and was performed through 1,000,000 generations by the Markov Chain Monte Carlo (MCMC) sampling trees every 100 generations. *Rosmarinus officinalis* was used as outgroup. Trees sampled from their posterior probability distribution were analyzed in order to observe the number of generations of trees needed to converge to a stable likelihood value for each separate data set (Huelsenbeck and Ronquist, 2001). Stability was achieved when the plotting of the log likelihood scores of sample points against generation time reached a stable equilibrium value (Leaché and Reeder, 2002).

Sampled points from generations previous to stationary were discarded manually, comparing the values of the logfiles and the tree files. All remaining trees were used to construct the respective 50% majority-rule consensus trees, where the percentage of times that a clade is recovered is interpreted as an estimation of robustness.

For the distance analyses, trees were constructed using the neighbor-joining method (NJ; Saitou and Nei, 1987) using proportional (p) Kimura two parameter in PAUP* and Mega softwares. Reliability of the trees was tested using 1,000 bootstrap and jackknife replications.

The results of the different inference phylogenetic methods within each data set were quite similar, so only selected results are presented here.

Results

Sequence analyses

The entire ITS region, including the 5.8S subunit and both spacers of the 29 species ranged to 735bp. The ITS region, of the 14 *Cunila* species ranged to 686bp excluding the 5.8S subunit that was obtained just for two species, *C. incana* and *C. galioides*. Because of the problems during PCR amplifications of the herbarium samples, we used the internal primers ITS3 and 74 (Desfeux and Lejeune, 1996). The entire ITS region were always analyzed together, as a single data collection. The proportion of C/G is greater than the proportion of A/T in each spacer. Most indels in the sequences are small (1-5 bp), being small inversions or duplications. Alignment of 29 sequences including 14 *Cunila* species, four *Agastache* Clayton ex Gronov. species, four *Lepechinia* Willd. species, one *Hyptis* Jacq. species, two *Glechon* Spreng. species, one *Hesperozygis*

Epling species and two *Hedeoma* Pers. species with the outgroup *R. officinalis* resulted in a matrix of 735 total characters with the introduction of indels. No evidence of obvious ITS length variants, in any of the accessions analyzed was observed.

Accessions, including out-groups, showed no extreme variation. The alignment to all sequences analyzed showed a very low variation within *Cunila* species.

The sequenced chloroplast region for 18 species including 13 *Cunila* species, two *Agastache* species, one *Hyptis* species and one *Hesperozygis* species with the out-group *R. officinalis* resulted in a matrix of 817 total characters with the introduction of indels., including the *trn*L intron and the *trn*L-*trn*F spacer. The indels sizes varied from a single base to 10 nucleotides. One important indel event was observed, corresponding to a deletion of 10bp shared by the whole *Cunila* species and *Hesperozygis spathulata*, being a synapomorphy of these species. The sequences showed several regions with repetitive bases, duplications and inversions, in addition, some indels could be attributed to sequence duplication.

Phylogenetic Analyses

The Partition Homogeneity Test (PHT) resulted in a value of 0.88 to the nuclear set x chloroplast set comparison, what meant incongruence non-significant, indicating significant level of congruence. For these reasons, the option of running the analyses in a combined matrix was followed. High levels of incongruence can result in polytomization or loss of phylogenetic information regarding one or more data sets, when these data are combined. In this case, running separated analyses to the incongruent data sets can improve the information available in the trees.

Parsimony based on heuristic searches with addition sequences as-is and random resulted in 287 equally parsimonious trees using the nuclear matrix, built with 160 parsimony-informative characters (CI=0.73, RI=0.60, RC=0.43; steps=569), two equally parsimonious trees for the chloroplast matrix, based on 55 parsimony-informative characters (CI=0.91, RI=0.72, RC=0.82; steps=228), six equally parsimonious trees for the combined matrixes, based on 171 parsimony-informative characters (CI=0.85, RI=0.74, RC=0.62; steps=556) and at last, ten equally parsimonious trees for the combined matrixes in the Exhaustive search (South American species only) based on 31 parsimony-informative characters (CI=0.91, RI=0.67, RC=0.61; steps=179). Sequence variation appear to be very low, despite this fact the datasets show an enough number of parsimony-informative characters to produce parsimony well resolved trees. This fact was evidenced by other authors, who worked with other genera belonging to Lamiaceae (El Qualid et al., 1999; Prather et al., 2002; Meimberg et al., 2006). The pattern and lack variation of the sequencing among species of *Cunila* can reflect a recent divergence time.

Phylogenetic trees produced from combined matrixes (Figures 6-7) are very similar to the trees produced by nuclear dataset, differing by the number of taxa. Unfortunately only 18 taxa were used in combined matrix because the disponibility of sequenced regions on GenBank. This combined matrix was composed by 13 new *Cunila* sequences and one new *Rosmarinus* sequence, and five GenBank sequences: one *Hesperozygis* species, one *Hyptis* species, two *Agastache* species. Figures 1a-2 shows phylogenetic trees produced from the nuclear dataset, Figures 3-5 shows phylogenetic trees based on chloroplast matrix and Figures 6-7 shows phylogenetic trees based on combined matrixes. Likelihood, neighbor-joining and Bayesian trees are presented

combined at the same figure, since the topologies are very similar. Numbers in branches indicate majority rule consensus and bootstrap values.

Each method tested was conducted with different parameters, resulting in similar or identical trees. The reliability of the resulting trees was tested using bootstrap (Felsenstein, 1985) and jackknife (Hedges, 1992) with 1,000 replications, in all methods the results obtained from bootstrap and jackknife were very similar or identical, being demonstrated here only the bootstrap results. The MP-exhaustive search, applied to the South American species resulted in a consensus tree very similar to these obtained for MP, when the same species are compared (data not shown), the reliability of these trees was tested using bootstrap and jackknife with 10,000 replications. The four phylogenetic methods applied generated similar consensus trees. Groups with bootstrap support were also similar among the different methods, however the trees generated based on distinct data sets presented few conflicts.

The South and North American *Cunila* species are separated by other genera in all analyses conducted based on plastidial, nuclear and combined matrix evidencing the paraphyly of the genus *Cunila*. The South American section *Spicatae* (subshrubs), defined by Epling (1936), are well-defined in all methods, exception is the MP consensus based on plastidial evidence (Figure 3). The other two South American sections defined by Epling (1936), *Incisae* and *Incanae* (shrubs), are very close related, confirming the data showed by ISSR molecular markers (Agostini et al., 2008). These two sections were found close related in all analyses conducted, composing the South American shrub group.

The Mexican species *C. polyantha*, *C. lythryfolia* and *C. pycnantha* clustered together in all nuclear analyses, forming a close related group with the USA species *C. origanoides* (Figures 1a-2). In the plastidial and combined analyses *C. pycnantha* was excluded because there were problems with DNA sequencing of herbarium material. Concerning the plastidial evidence, the North American group was formed in the ML, BI and NJ analyses (Figures 4-5), but the MP consensus tree showed only the Mexican species grouped together (Figure 3). All combined analyses showed North American species grouped together (Figures 6-7).

Discussion

This is the first phylogenetic study using molecular data on the genus *Cunila*. The nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast *trn*L-*trn*L-*trn*F region have proven to be useful in postulating phylogenetic hypotheses and refining the systematics for several genera of Lamiaceae (El Qualid et al., 1999; Steane et al., 1999; Lindqvist e Albert, 2002; Prather et al., 2002; Bunsawat et al., 2004; Walker et al., 2004; Walker and Sytsma, 2007; Meimberg et al., 2006; Edwards et al., 2006; Rosselló et al., 2006; Oliveira et al., 2007), being both datasets useful for this *Cunila* phylogenetic study.

Occurrence of polymorphic ITS repeats, due to a failure in full concerted evolution has been usually invoked as evidence of hybridization introgression and/or polyploidization events among taxa displaying different ribotypes (O´Kane et al., 1996; Campbell et al., 1997; Brasier et al., 1999; Rauscher et al., 2002). However, a detailed inspection of direct and reverse nuclear sequences from *Cunila* species does not show

evidence that the polymorphism found is due to interspecific hybridization, which has never been reported to this genus.

The molecular results presented here highlight some systematic questions within the genus *Cunila*, particularly the closely related similarity observed between the South American sections *Incisae* and *Incanae*.

The paraphyly of the genus *Cunila* are in evidence in all analyses. With the exception of MP consensus based on cpDNA, results obtained from nuclear, plastidial and combined sets support a division of the genus *Cunila* into two paraphyletic groups, one belonging to the North America and other belonging to the South America.

The South and North American *Cunila* species were divided in two groups by the inclusion of *Glechon marifolia*, *Glechon thymoides*, *Hesperozygis spathulata*, *Hedeoma costata* and *Hedeoma apiculata*, in all analyses conducted based on nuclear matrix evidencing the paraphyletic status of the genus *Cunila*. The South American subshrubs (*Spicatae*) and shrubs (*Incisae* and *Incanae*) sections were well-defined in all nuclear analyses but showing low bootstrap value (Figures 1a-2). The shrubs *C. incana* (*Incanae*) and *C. angustifolia* (*Incisae*) grouped together with the highest bootstrap value (96% - Figure 1a), these data strengthen the inclusion of these two species in a single section. The North American species grouped together in a cluster divided in two subgroups: first composed by the Mexican species strongly supported by bootstrap value (100%) and the second composed by *C. origanoides* representative of the U.S.A (Figures 1a-2).

Plastidial analyses were performed with only 18 species because the availability of sequences. Maximum Parsimony consensus tree based on plastidial fragment showed two principal clusters, one formed by *Hyptis emoryi* and *Agastache* species, and other

composed by all *Cunila* species and *H. espathulata* with high bootstrap value (99% - Figure 3). This group was characterized by the presence of five subgroups: South American shrubs (*Incisae* and *Incanae* sections - 96% bootstrap value); *C. menthoides* and *H. spathulata*; *C. origanoides* and *C. menthiformis*; Mexican species (*C. polyantha* and *C. lythrifolia* - 99% bootstrap value) and *C. platyphylla* and *C. fasciculata*, this last subgroup was formed in all analyses with high bootstrap support. The inclusion of *H. spathulata* in this *Cunila* group can be an evidence of the paraphyly reported in the other analysis. Plastidial analysis based on ML, BI and NJ (Figures 4-5) separated the South and North American species in two distinct groups strongly supported by bootstrap value, showing an interesting fact, the inclusion of *H. spathulata* inside the South American *Cunila* group with moderated bootstrap value. In this case *H. spathulata* are plotted between the South American subgroups: shrubs and subshrubs.

In a study of staminal evolution in the genus *Salvia*, a representative of *Spicatae* section (*C. galioides*) and a representative of *Incanae* section (*C. incana*) were used within a lot of other genera to build a phylogeny (Walker and Sytsma, 2007). In this work, *C. galioides* formed a subgroup with *G. marifolia* and *G. thymoides* while *C. incana* were plotted in a polytomy with other genera. This facts point to a possible paraphyletic status of the South American *Cunila* group, but to verify this hypothesis more research is needed with a larger number of *Glechon* and *Hesperozygis* samples.

A combined dataset were composed by the species that had both fragments sequenced, being new sequences or GenBank sequences. The analyses using the combined dataset resulted in trees very similar to nuclear evidences. This fact can be attributed to the most number of informative characters found in ITS fragment. In all

analyses are in evidence two groups: the first one is composed by *H. emoryi* and *Agastache* species; and the other is composed by *H. spathulata* and *Cunila* species (99% bootstrap value). The *Cunila* cluster was separated in two groups with the inclusion of *H. spathulata*: South and North American groups. The *Cunila* South American group according with nuclear and plastidial analysis was composed by the subshrubs and shrubs subgroups. Mexican species grouped together with high bootstrap values (98%), forming a cluster with *C. origanoides*, representing of U.S.A. (92%).

In the MP-exhaustive search two groups were in evidence: the group composed by the subshrubs species (*Spicatae* section) and the group composed by shrub species (*Incisae* and *Incanae* sections), both well-defined showing high (95) bootstrap values (data not shown).

The cluster formed by the North American species *C. origanoides* and the South American species *C. menthiformis* (*Spicatae* section) in MP consensus based on chloroplast analyses (figure 3) is weakly supported, whereas in all other analysis the species *C. menthiformis* and *C. microcephala* grouped together with high support. This species belong to *Spicatae* section and show very similar morphologies being found normally in swamps, supporting their long branches on other plants. *Cunila origanoides* appear as monophyletic with the North American species, in nuclear, plastidial and combined datasets. The species *C. platyphylla* and *C. fasciculata* grouped together in all analyses with high or moderate support. This species belong to the *Spicatae* section but not show evident morphology similarities.

The cluster composed by South American shrub species was in evidence in all analyses based on the nuclear, plastidial and combined dataset with high and moderated

bootstrap support. The most interesting point is the fact that the species *C. angustifolia* and *C. incana*, belonging to the *Incisae* and *Incanae* sections respectively, are closer related than the species *C. angustifolia* and *C. incisa*, both belonging to the same *Incisae* section. The group closely related formed by these three shrub species are strongly supported by this paper and the data obtained by molecular markers ISSR (Agostini et al., 2008), but are in contradiction with the first classification suggested by Epling (1936) who differ the section *Incanae* from the other sections by the presence of single flowers in upper axils. Based on data and support obtained in all analyses presented here, we suggest the union of the two South American sections formed by shrubs species (*Incisae* and *Incanae* sections) with new nomenclature to this reformulated section and a taxonomical study to rename the South American *Cunila* group.

All analyses presented here points to the paraphyletic status of the genus *Cunila*, and in some analyses the South American sections seem to be paraphyletic, but the inclusion of other representatives of *Hesperozygis* and *Glechon* are needed.

Phylogenetic hypotheses should be based on as many characters as possible, but the phylogeny presented here is estimated based in sequences from two DNA regions, being the first report about phylogenetic relationship within *Cunila* species. The results presented here about the relationship of the South American species confirm data showed by molecular marker ISSR (Agostini et al., 2008). Meanwhile, for *Cunila* these regions showed low sequence divergence among species, possible reflecting recent evolution, despite this fact the datasets show an enough number of parsimony-informative characters to produce parsimony well resolved trees. The phylogenetic hypothesis presented here yielded considerable insight about the evolution of *Cunila*, which is

strongly supported as paraphyletic. Future works will focus on different approaches to better clarify the phylogeny of *Cunila,* identifying low copy nuclear genes that may be more variable and therefore yield greater phylogenetic resolution, as well other molecular markers, chemical and cytogenetic evidences.

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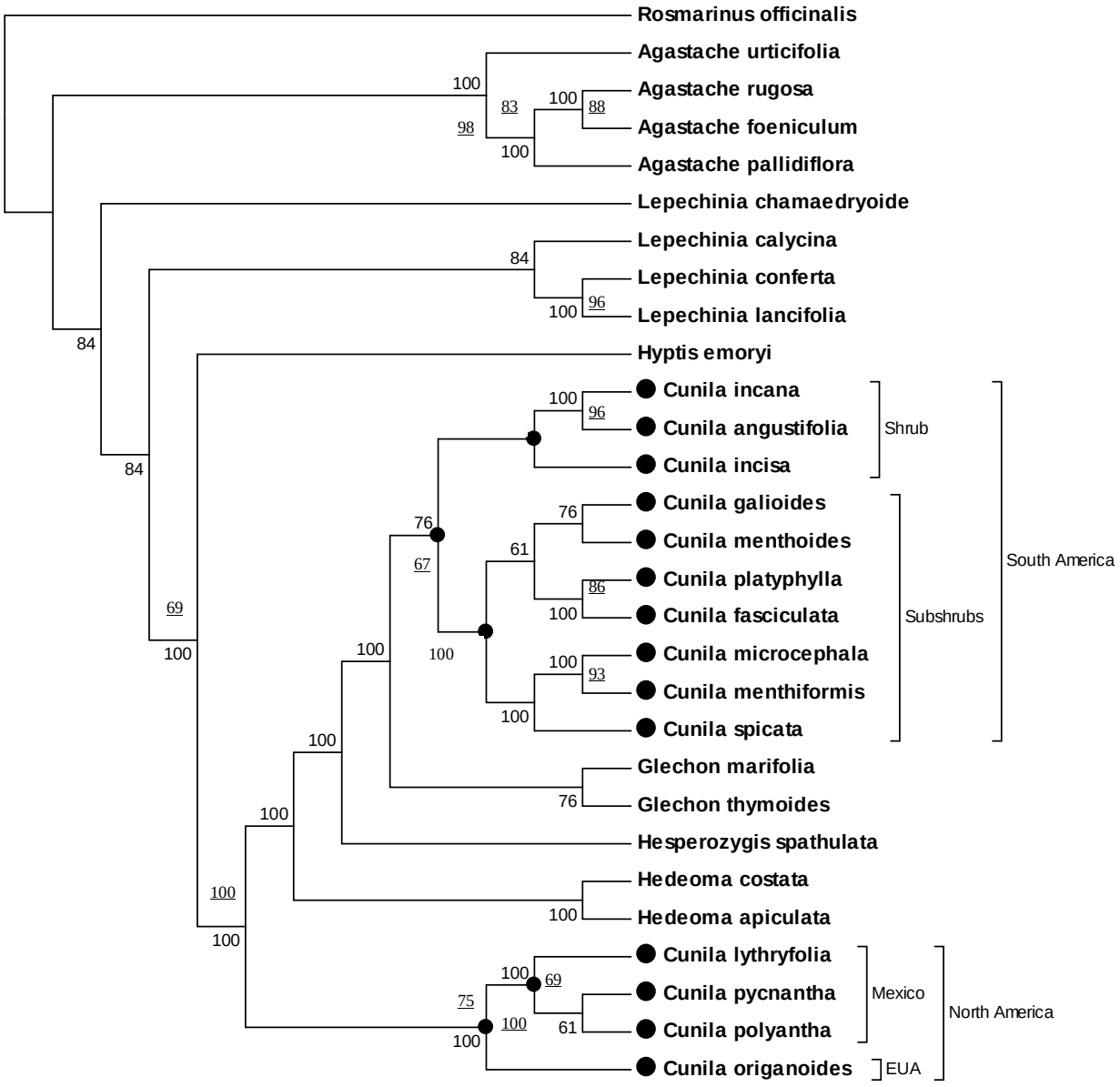

Figure 1a: ITS dataset parsimony consensus: values above and bellow branches corresponding to 50% majority rule and underscore values corresponding to bootstrap.

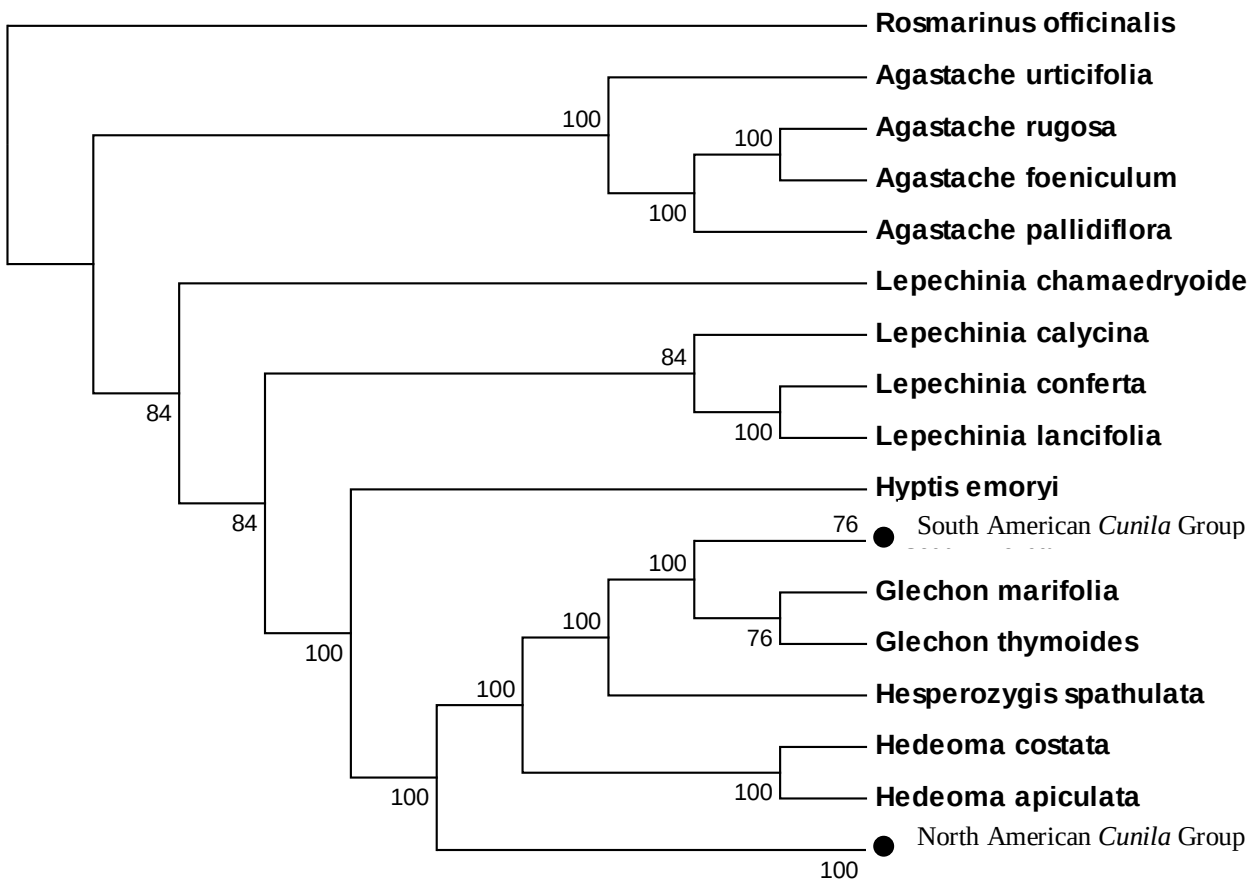

Figure 1b: ITS dataset parsimony consensus resumed tree: values above and bellow branches corresponding to 50% majority rule.

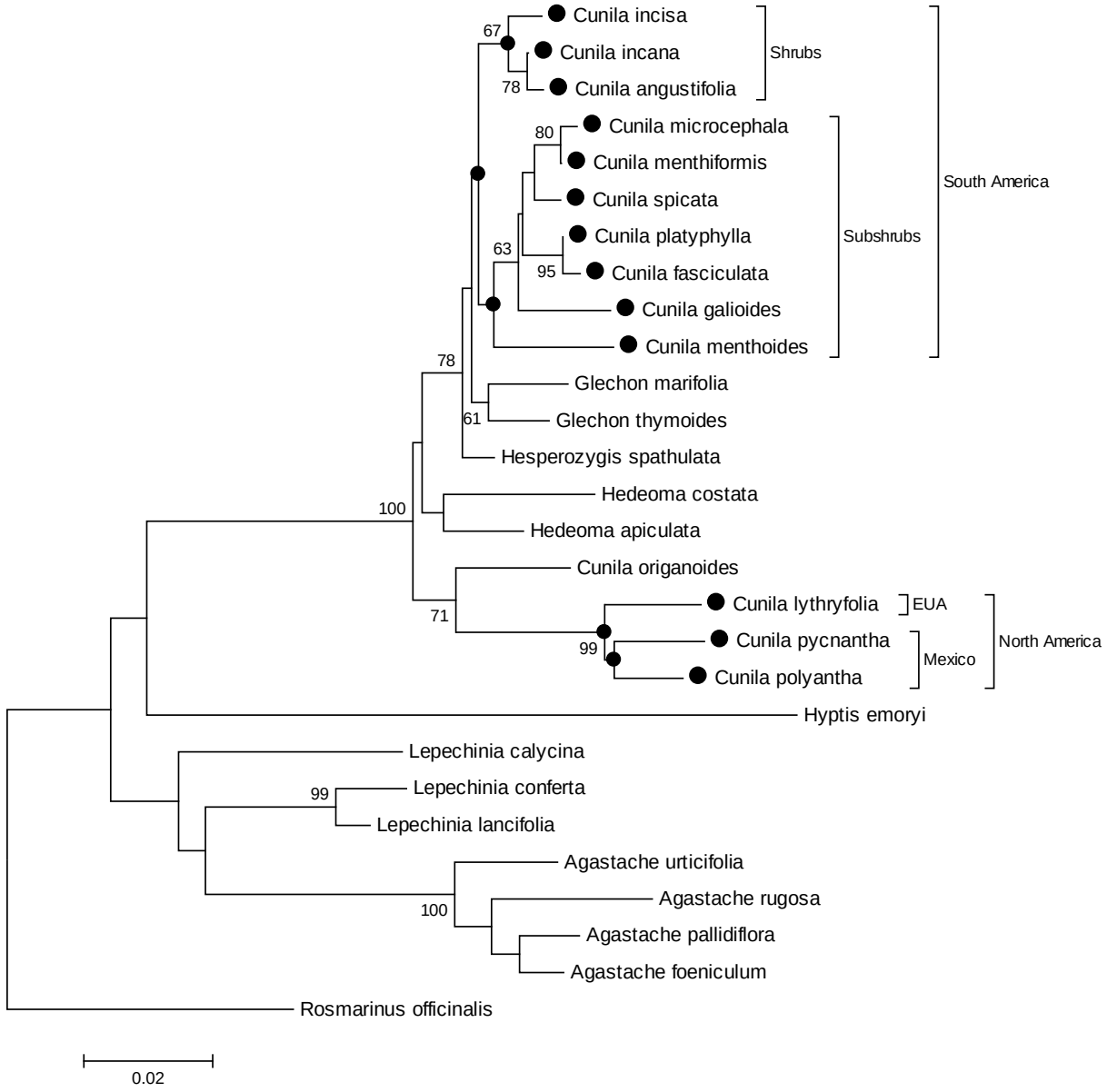

Figure 2: ITS dataset Maximum Likelihood, Bayesian inference and Neighbor-Joining combined tree: branches values corresponding to bootstrap (> 60%).

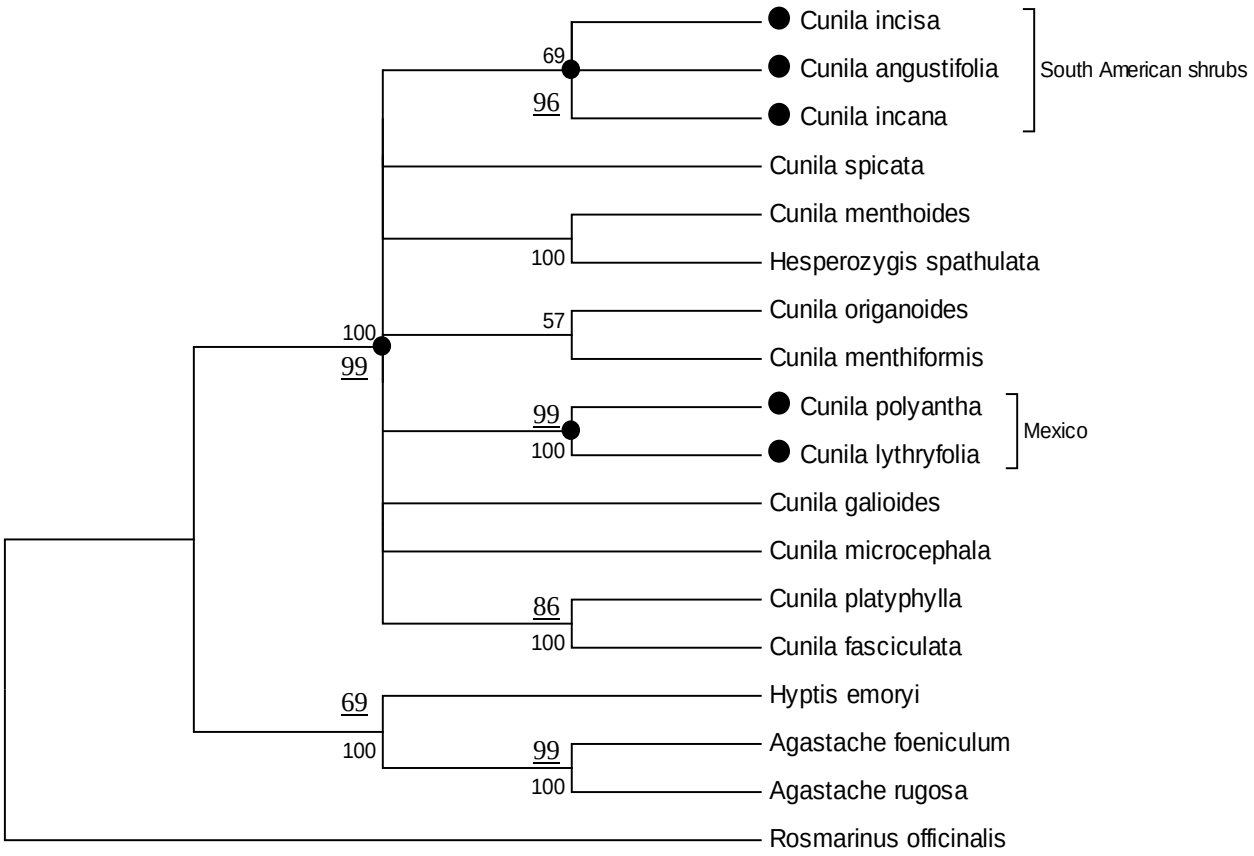

Figure 3: Maximum Parsimony consensus tree based on cpDNA evidence: values above and bellow branches corresponding to 50% majority rule and underscore values corresponding to bootstrap values.

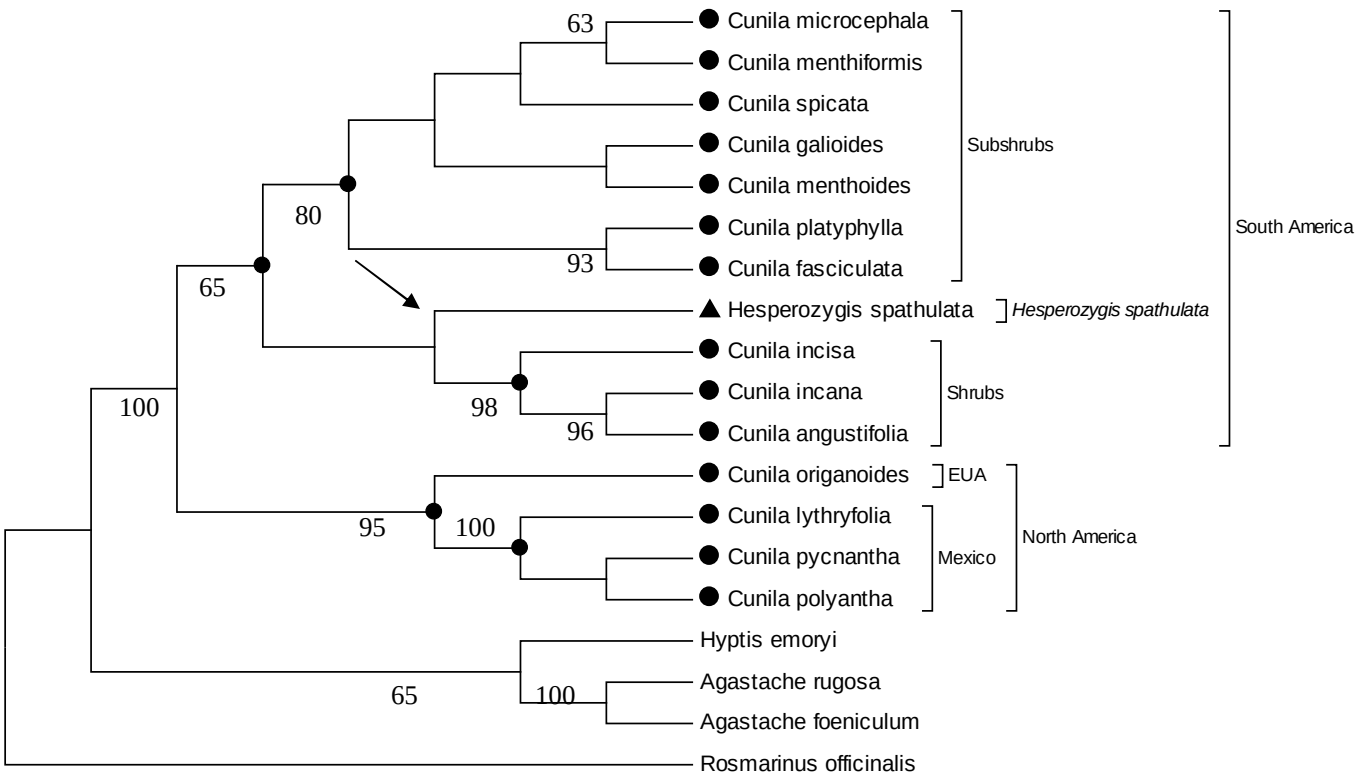

Figure 4: Maximum Likelihood consensus and Bayesian inference tree based on cpDNA evidence: values on branches corresponding to bootstrap (> 60%). Arrow representing the *Hesperozygis spathulata* insertion point.

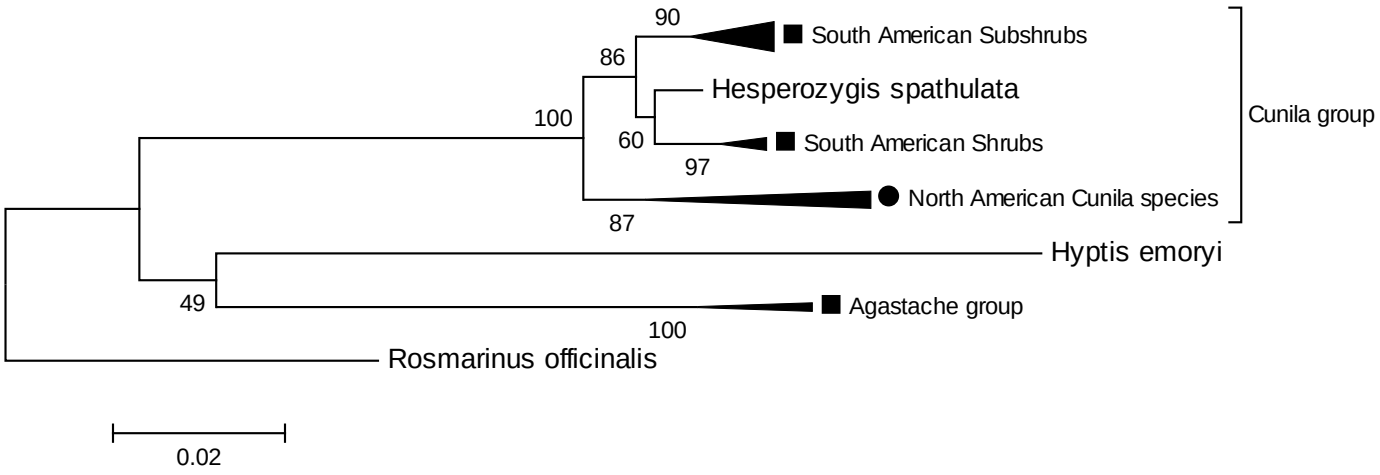

Figure 5: Neighbor-Joining tree based on cpDNA evidencing the separation of the Shrub and Subshrub groups of South American species of *Cunila*: branches values corresponding to bootstrap.

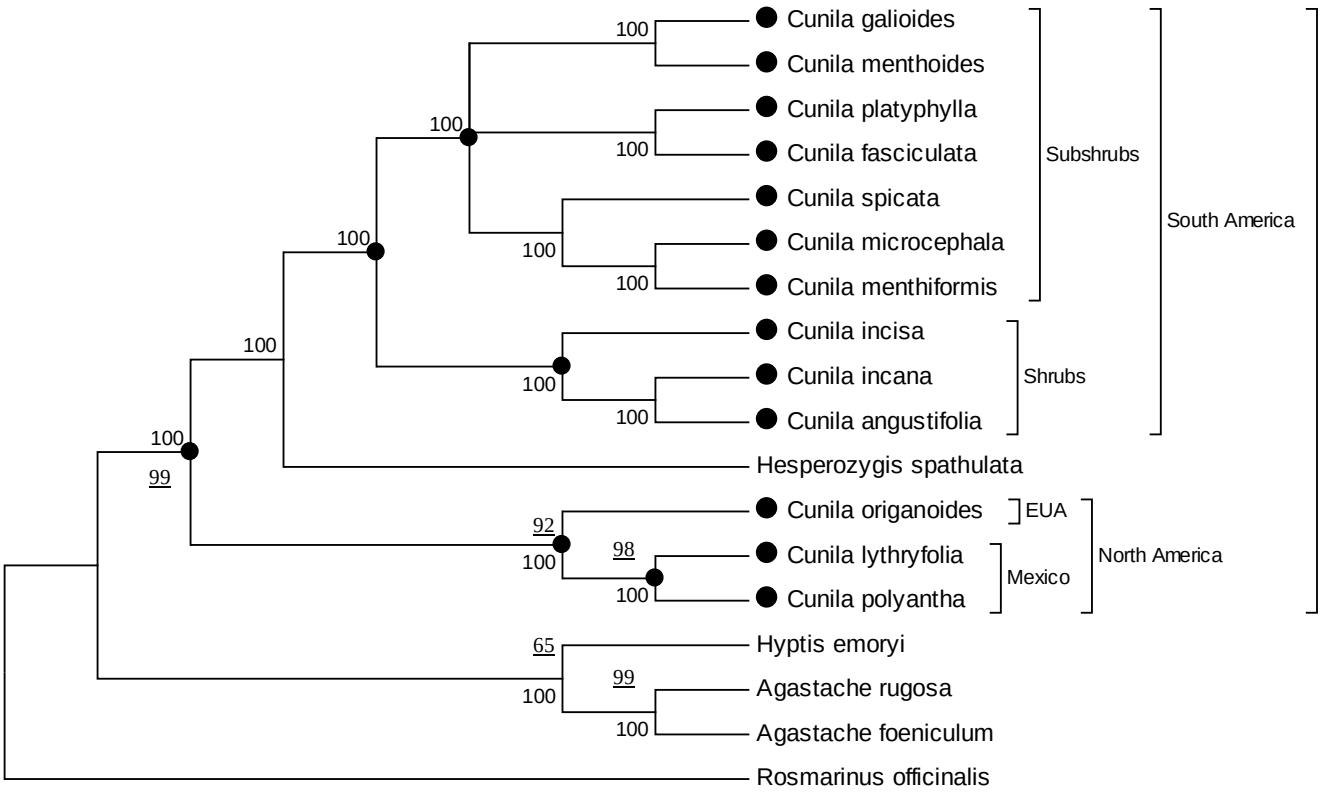

Figure 6: Combined dataset parsimony consensus: values above and bellow branches corresponding to 50% majority rule and underscore values corresponding to bootstrap values.

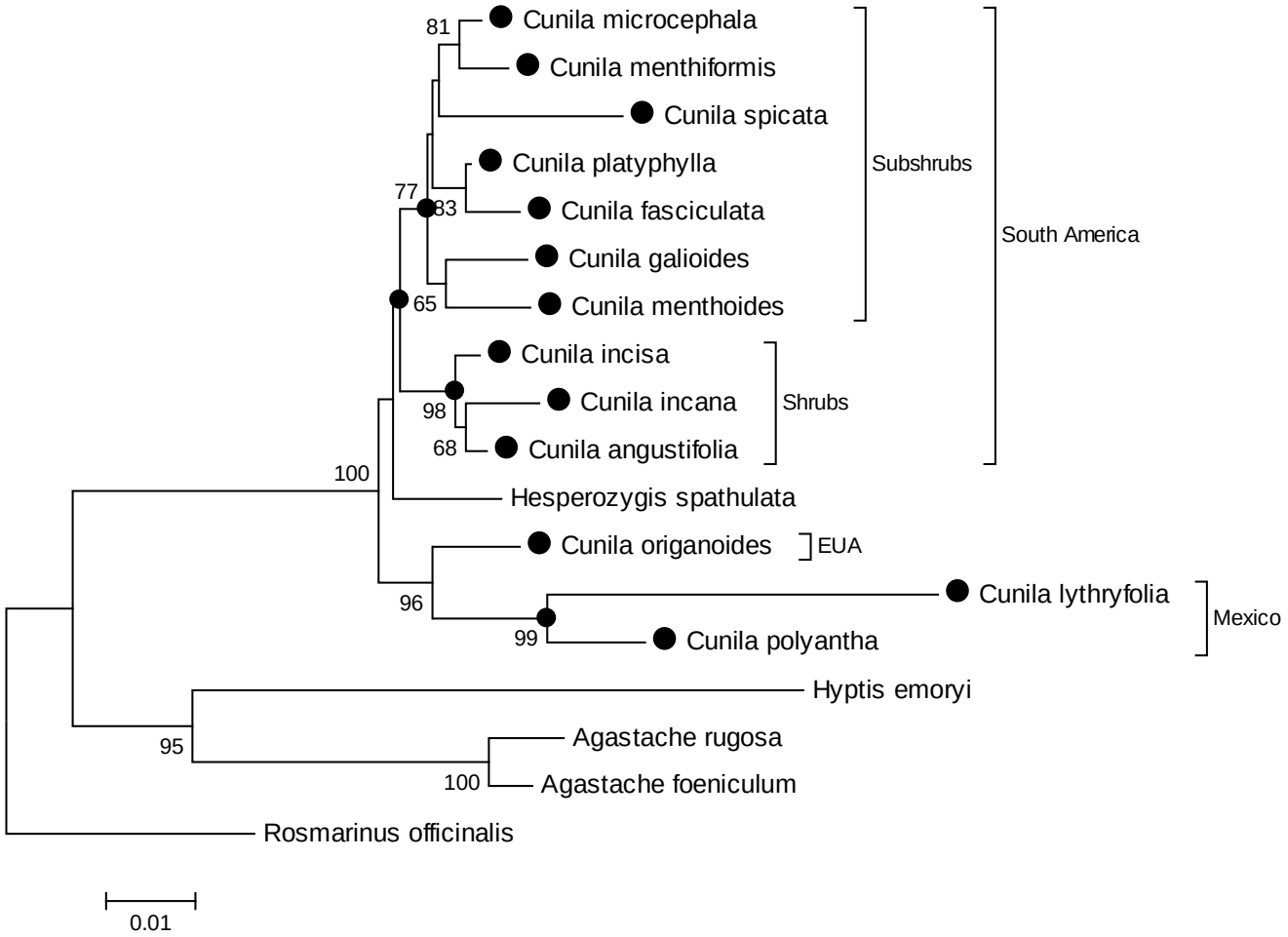

Figure 7: Combined dataset Maximum Likelihood, Bayesian inference and Neighbor Joining combined tree: values on branches corresponding to bootstrap values.

Table 1: Plant samples from each species of *Cunila* collected at multiple locations in Rio Grande do Sul State, herbarium number of received species and GenBank accession numbers.

Taxon	Locality	Latitude	Longitude	GenBank accession number *trn*L-*trn*L-*trn*F	GenBank accession number ITS
Cunila galioides Benth.	Bom Jesus	S 28°38´39´´*	W 50°38´14´´*	-	-
Cunila spicata Benth.	Esmeralda	S 28°03´13´´*	W 51°11´25´´*	-	-
Cunila angustifolia Benth.	Cambará do Sul	S 29°02´52´´*	W 50°08´41´´*	-	-
Cunila microcephala Benth.	Canguçu	S 30°53´13.9´´	W 52°14´58.3´´	-	-
Cunila menthiformis Epl.	Esmeralda	S 28°03´13´´*	W 51°11´25´´*	-	-
Cunila incisa Benth.	Veranópolis	S 28°56´10´´*	W 51°32´8´´*	-	-
Cunila menthoides Benth.	Caçapava do Sul	S 30°32´28.2´´	W 53°33´18.7´´	-	-
Cunila platyphylla Epl.	Vacaria	S 28°30´44´´*	W 50°56´02´´*	-	-
Cunila incana Benth.	Caçapava do Sul	S 30°30´44´´ *	W 53°29´29´´*	-	-
Cunila fasciculata Benth.	ICN 35910	-	-	-	-
Cunila origanoides (L.) Britton	MIN 67984	-	-	-	-
Cunila lythrifolia Benth.	MIN 13989	-	-	-	-
Cunila polyantha Benth.	MIN 6089	-	-	-	-
Cunila pycnantha B.L. Rob. & Greenm.	MIN 1905	-	-	-	-
Rosmarinus officinalis L.	UCS-Caxias do Sul (Cultivated)	S 29°10´05´´*	W 51°10´46´´*	-	-
Agastache urticifolia (Benth.) Kuntze	-	-	-	-	DQ667247.1
Agastache rugosa (Fisch. & C.A. Mey.) Kuntze	-	-	-	EU244612.1	DQ132861.1
Agastache foeniculum Kuntze	-	-	-	AY506626.1	AY506660.1
Agastache pallidiflora (A. Heller) Rydb.	-	-	-	-	AY771704.1
Lepechinia chamaedryoides Epl.	-	-	-	-	DQ667232.1
Lepechinia calycina Epl. ex Munz	-	-	-	-	DQ667308.1
Lepechinia conferta (Benth.) Epl.	-	-	-	-	DQ667307.1

Lepechinia lancifolia (Rusby) Epl.	-	-	-	-	DQ667306.1
Hyptis emoryi Torr.	-	-	-	AY506629.1	AY506664.1
Glechon marifolia Benth.	-	-	-	-	DQ667303.1
Glechon thymoides Spreng.	-	-	-	-	DQ667310.1
Hesperozygis spathulata Epl.	-	-	-	AY506602.1	AF369166.1
Hedeoma costata A. Gray	-	-	-	-	DQ667236.1
Hedeoma apiculata W.S. Stewart	-	-	-	-	AY771706.1

* Coordinates of nearby cities.

CAPÍTULO II

Variabilidade genética do gênero *Cunila* D. Royen ex L.

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Genetic relationships among South American species of *Cunila* D. Royen ex L. based on ISSR

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Abstract: *Cunila* species are among the commonly used South Brazilian plants in folk medicine. This genus presents two centers of distribution in North and South America; the latter being classified into three sections: *Incanae, Incisae* and *Spicatae*. Based on the prospective utility as aromatic and medicinal plants, the aim of this work was to examine the genetic diversity among the South American species to contribute to the knowledge of their botanical sections. In this context, eleven *Cunila* species were analyzed by ISSR using seven primers that generated a total of 107 bands. The relationship was evaluated constructing dendrograms using the UPGMA algorithm and analysis of principal components. The cluster analysis places the species *C. origanoides* with the South American species, but forming an independent cluster. Remarkably, among South American species two clusters emerge, one consisting of shrubs and the second by subshrubs species, which refines the botanical taxonomy for several species of the genus.

Key words: *Cunila*; aromatic and medicinal plants; ISSR; botanical sections; interespecific diversity.

Introduction

The genus *Cunila* D. Royen ex L. belongs to the family Lamiaceae, subfamily Nepetoideae, and tribe Mentheae (Cantino *et al*. 1992). This genus presents two centers of distribution, one in North America formed by nine species and another in the southern regions of South America with eleven species (Garcia-Peña 1989; Bordignon, 1997). Both northern and southern species of *Cunila* are used in popular medicine as stimulants, aromatics, antispasmodics, emenagogues, antithermics and in the treatment of chronic coughs and respiratory infections (Simões *et al*. 1994). Moreover, *Cunila* essential oils have compounds responsible for antibacterial, antifungal and insecticidal activity (Duke 1994).

Based on growth characteristics and inflorescence morphology, Epling (1936) classified the South American species into three sections: *Incanae, Incisae* and *Spicatae*. The section *Incanae* has only one species, *C. incana* Benth. typically shrubs with single pale yellow hairy flowers at upper axils. The section *Incisae* comprises two species, *C. incisa* Benth. and *C. angustifolia* Benth., both shrubs with small glabrous white inflorescences. The third section, *Spicatae*, is the most important in number of species, comprising seven: *C. menthoides* Benth., *C. platyphylla* Epling, *C. menthiformis* Epling, *C. spicata* Benth., *C. galioides* Benth., *C. fasciculata* Benth., *C. microcephala* Benth. and *C. tenuifolia* Epling. This section is characterized by subshrubs or evergreen perennial herbs with terminal spikes or globular inflorescences formed by small white fairly glabrous flowers.

Despite their economic potential, the genus *Cunila* have been poorly studied genetically. The taxonomy of the genus, particularly for the case of South American species, is restricted to morphological analyses made by Epling in the mid 20th century

(Epling 1936) and more recent chemotaxonomic data (Bordignon *et al*. 1996, 1997, 1998, 1999, 2003).

Molecular markers developed during the last two decades have largely overcome the problems associated with phenotype-based classification in several plants. Firstly, isozymes and Restriction Fragment Length Polymorphisms (RFLP) were used as reliable markers for genetic analysis in plants (Botstein *et al*. 1980). More recently, PCR based techniques such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeats (SSR), provided faster, easy to handle and less expensive approaches. Among these, RAPD has been widely used to evaluate intraspecific variations in several aromatic and medicinal species from the Lamiaceae family, such as *Salvia fruticosa* Mill. (Skoula *et al*. 1999), *Thymus vulgaris* L. (Echeverrigaray *et al*. 2001) and *C. galioides* (Fracaro *et al*. 2005). However, this method bears reproducibility problems that limit its use. Conversely, ISSR markers use longer primers that ensure higher reproducibility (Bornet and Branchard 2001, Reddy *et al*. 2002), and have a higher predisposition to reveal polymorphisms offering a great potential to determine intra- and interspecific levels of variation. In addition, ISSR are particularly advantageous to analyze unknown genomes when there is a lack of genomic information of the studied taxa (Zietkicwicz *et al*. 1994, Shi *et al*. 2006) and the ISSR-target sequences are abundant throughout the genome of eukaryotes, evolving quickly (Fang and Roose 1997, Esselman *et al*. 1999). Moreover, ISSR are particularly advantageous to analyze taxa for which there is scarce genomic information (Zietkicwicz *et al*. 1994, Shi *et al*. 2006), and in addition quickly evolving eukaryotes can be analyzed if the ISSR-target sequences are abundant throughout their genome (Fang and Roose 1997, Esselman *et al*. 1999). Within the family Lamiaceae, ISSR

banding patterns were used to examine genetic diversity within and among populations of *Monarda fistulosa* L. var. *brevis* Fosberg & Artz (Kimball *et al*. 2001), *Hesperozygis ringens* (Benth.) Epling (Fracaro and Echeverrigaray, 2006), *Cunila spicata* Benth. (Albuquerque 2004) and *Lamiophlomis rotata* Benth. ex J. D. Hooker (Liu *et al*. 2006).

Based on the potential of this genus as aromatic and medicinal plants, the objective of the present study was to examine the genetic diversity among the South American species of *Cunila*, contributing to the knowledge of its botanical sections, the genetic relationships among their species and the genetic variation within this genus.

Material and methods

Plant material

Samples from each species of *Cunila* (except for *C. fasciculata*) were collected at different locations in Rio Grande do Sul state (Table 1) between September 2005 and April 2006. *Cunila fasciculata* is included in the official red list of endangered species of Rio Grande do Sul state (http://www.sema.rs.gov.br/sema/html/pdf/especies-ameacadas.pdf), and not surprisingly the species was not found during the expeditions. In this case, we used a dried sample from the herbarium ICN (ICN 35910). One herbarium sample of *C. origanoides* (L.) Britton (MIN 67984) was included to estimate the genetic divergence between North and South American species. Samples of *Mentha arvensis* L. and *Rosmarinus officinalis* L., both belonging to family Lamiaceae, were also included in the analysis as out-groups. The plants used in this study were identified by Dr. Sérgio A. de L. Bordignon, and a specimen of each species was deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (Brazil).

DNA extraction

Equal amounts (0.1g) of leaf tissue from each sample were prepared and dried with silica gel, placed in porcelain mortars and frozen with liquid nitrogen to be ground to a fine powder. Total genomic DNA was extracted by the CTAB modified method described by Doyle and Doyle (1987). DNA samples were quantified in spectrophotometer at 260 nm. The $A_{260/280}$ reading to each DNA ranged from OD 1.8 to 2.0.

PCR amplification

PCR amplifications were performed in a total reaction volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM each dNTP, 1.15 mM $MgCl_2$, 0.1 μM of primer, 1.5 unit of Taq polymerase (Cenbiot), 2% of DMSO and 50 ng of genomic DNA. DNA amplifications were performed using a TECHNE TC-412 thermal cycler. The amplification conditions consisted of one initial 5 minutes step at 92 °C followed by 35 cycles of 94 °C (1 min), 45s for annealing temperature (48 °C/50 °C), and 72 °C (2 min), the reactions were completed by a final extension step of 5 min (72 °C) and 5 min (4 °C).

The ISSR amplification products were stained by Syber Green (0.5 mg/mL) and separated by horizontal electrophoresis in 1.5% agarose gel soaking in 1X TBE buffer (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) at 70 mA.

The electrophoresis gels were visualized and photographed under ultra violet light. The size of the amplified products was determined by comparison with 100 bp Ladder molecular weight (Amersham Biosciences).

Six anchored oligonucleotide primers $(CA)_8G$, $(CTC)_4RC$, $(AC)_8T$, $(AG)_8A$, $(GA)_8T$, $(AG)_8YC$, and one free primer, $(GACA)_4$, were used to amplify all samples.

These primers were selected from a set of 20 ISSR primers based on the number of amplification products and the quality of the profiles obtained with five samples: *C. incisa, C. menthoides, C. platyphylla, C. galioides* and *M. arvensis*.

Statistical analysis

Bands were scored as a binary variable, for presence (1) or absence (0) of each fragment size. The binary matrix (1/0) was used to calculate the similarity by Dice coefficient among each pair of samples. All data for the three samples from each species were combined to calculate the Dice similarities showed in Table 3. Amongst the various similarity indices, the Dice coefficient was chosen as the most appropriate for dominant markers like ISSR and RAPD, since it does not attribute any genetic meaning to the coincidence of absent bands. The Dice coefficient was calculated with the formula: $2N_{AB}/(2N_{AB} + N_A + N_B)$, where N_{AB} is the number of bands shared by samples, and N_A and N_B represent the amplified fragments in samples A and B, respectively.

The relationship among species was evaluated constructing dendrograms by the UPGMA (Unweighted Pair Group Method Using Arithmetic Averages) algorithm, and analysis of principal components. These statistical analyses were performed using NTSYS package (Rohlf, 2001) and SPSS 10.0 software (SPSS Inc., Chicago, Illinois). The permutation analysis (1000 permutations) was performed using WinBoot (Yap and Nelson 1996) and the AMOVA analysis was conducted with the GenAlEx program (Peakall and Smouse 2001).

The discrimination potential of each primer was expressed by Simpson's coefficient ($h_j = \Sigma\ (1- \Sigma p_i^2)/n$), where p_i is the frequency of the i^{th} allele, and n corresponds to the number of loci detected by each primer (Hunter and Gaston 1988,

Valk *et al*. 2005). A value of 1.0 indicates that the primer is able to discriminate between all samples and a value of 0.0 indicates that all samples are identical.

Results and discussion

To guarantee the suitability of ISSR primers, twenty primers were screened against total DNA from five samples: *C. incisa*, *C. menthoides*, *C. platyphylla*, *C. galioides* and *M. arvensis*. Based on the number of amplification products, the quality of the profiles, the level of polymorphism and the reproducibility, seven primers were selected for the identification of *Cunila* species and evaluation of their relationship. When applied to all *Cunila* samples (31), these primers displayed high discriminating ability, substantiated by Simpson's indexes ranging from 0.596 to 0.921, averaging 0.853 (Table 2), which demonstrates that a small number of primers is sufficient for this methodology.

When applied to the 31 plant samples under study, the seven selected primers generated a total of 107 amplified fragments with an average of 15.3 fragments per primer. The size of the amplified products ranged from 150 to 1700 bp and the number products per primer varied from 9 [$(CA)_8G$) to 24 ($(AC)_8T$]. We observed 97.2% (104) of the bands were polymorphic, including *M. arvensis* and *R. officinalis* in the analysis. Moreover, considering only the species of the genus *Cunila*, the percentage of polymorphic bands was 96.3%, and excluding the North American species (*C. origanoides*), the percentage was 95.3%. Thus, as expected, the percentage of polymorphism observed among species of *Cunila* was higher than that previously reported with RAPD and ISSR markers among populations of *C. galioides* (Fracaro *et al*. 2005), *C. incisa* (Agostini 2003) and *C. spicata* (Albuquerque 2004).

A unique pattern of amplified fragments was obtained for each species with only few differences. Moreover, these patterns of amplification revealed species-specific markers for *Cunila*. For instance, the primer $(GACA)_4$ amplified a fragment of 600 bp that characterized all the species of the genus *Cunila*. Primers $(CTC)_4RC$, $(GACA)_4$, and $(AC)_8T$ revealed fragments of 680 bp, 950 bp, and 500 bp, that were characteristic of the South American shrub *Cunila* species, *C. incisa*, *C. angustifolia* and *C. incana*, respectively. Such species-specific ISSR markers have been documented in other genus, like *Vigna* (L.) Hepper (Ajibade *et al*. 2000), *Cicer* L. (Sudupak 2004), *Lycoris* Herb. (Shi *et al*. 2006) and *Stachys* L. (Kochieva *et al*. 2006).

Genetic proximity inferred from Dice's similarity index for all pair of species varied from 0.214 to 0.788 (Table 3). The interspecific genetic similarity was considerably high (>0.964), confirming data obtained for some *Cunila* species using RAPD (Agostini 2003; Fracaro *et al*. 2005) and ISSR markers (Albuquerque 2004).

The highest similarity values between species were obtained for the comparison between *C. microcephala*/*C. menthiformis* (0.788), *C. incisa*/*C. angustifolia* (0.685), *C. incisa*/*C. incana* (0.682) and *C. menthoides*/*C. platyphylla* (0.632) and, as expected, the lowest similarity values were obtained between *Cunila* species and the out-group members of the same tribe, genera *Mentha* and *Rosmarinus*, that presented an average distance of 0.334 and 0.284 to *Cunila*, respectively.

The cluster analysis based on the ISSR results, was used to construct a UPGMA dendrogram (Figure 1), which distinctly separated the eleven *Cunila* species from the out-group species (*M. arvensis* and *R. officinalis*). The species *C. origanoides* clustered with the South American species, but forms an independent cluster, indicating that this species is a member of the genus *Cunila*, but belongs to a separated genetic group.

In regards to South American species, two clusters appear in the dendrogram (Figure 1). The larger one is formed by *C. galioides*, *C. spicata*, *C. fasciculata*, *C. menthiformis*, *C. menthoides*, *C. platyphylla*, and *C. microcephala*, and the smaller is formed by *C. incisa*, *C. angustifolia* and *C. incana*. These clusters present an average similarity of 0.441 ± 0.052 between them. The separation of these groups among *Cunila* species was confirmed by AMOVA analysis of variance and principal component analysis (Figure 2).

The first group includes all the subshrubs species of the *Spicatae* section with an average similarity of 0.515 ± 0.089. The *Spicatae* section is botanically defined as herbaceous perennial plants or subshrubs with sessile flowers consisting of terminal or sub-terminal spikes or globular inflorescences. The second cluster group is constituted by the three species of the genera classified as sections *Incanae* and *Incisae*, with an average similarity of 0.653 ± 0.052. These two sections include shrubs species and are botanically differentiated by the presence of single flowers or inflorescences at leaves axiles and the presence or absence of hairy trichomes on the leaves (Epling 1936). However, the high genetic similarity observed among *C. incisa*, *C. angustifolia* and *C. incana* in the present work does not support this separation into two different sections. Furthermore, chemical analysis of the essential oils of these species has shown that all of them accumulate considerable amounts of flavonoid aglycones, a group of compounds that is seldom observed among the members of the *Spicatae* section (Bordignon *et al*. 2003).

In addition, the *Spicatae* cluster presented more internal variation than the shrub species of the genus *Cunila* (Figure 1). This cluster can be divided into three subclusters: (1) *C. microcephala* and *C. menthiformis*, (2) *C. menthoides* and *C.*

platyphylla, and (3) *C. galioides*, *C. spicata* and *C. fasciculata*, and these subgroups are in accordance with the morphological characteristics of these species (Bordignon 1997).

To conclude, the molecular analysis of *Cunila* species based on ISSR markers refined the botanical taxonomy formerly accepted in systematics for several species of the genus. For instance, representatives of the *Incisae* and *Incanae* botanical sections were clustered in a single genetic group, clearly separated from the species of the *Spicatae* section. Moreover, *C. origanoides* grouped with other *Cunila* species, but formed a separate cluster, indicating that North and South American *Cunila* species represent different genetic pools.

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Table 1. Plant samples from each species of *Cunila* collected at multiple locations in Rio Grande do Sul State.

Species	Location	Latitude	Longitude	Nº of samples
Cunila galioides Benth.	Bom Jesus	S 28°38´39´´*	W 50°38´14´´*	2
Cunila galioides Benth.	Vacaria	S.28°03´40´´*	W 50°56´18´´*	1
Cunila spicata Benth.	Esmeralda	S 28°03´13´´*	W 51°11´25´´*	3
Cunila angustifolia Benth.	Cambará do Sul	S 29°02´52´´*	W 50°08´41´´*	3
Cunila microcephala Benth.	Canguçu	S 30°53´13.9´´	W 52°14´58.3´´	3
Cunila menthiformis Epling	Esmeralda	S 28°03´13´´*	W 51°11´25´´*	3
Cunila origanoides (L.) Britton	MIN 67984			1
Cunila incisa Benth.	Veranópolis	S 28°56´10´´*	W 51°32´8´´*	3
Cunila menthoides Benth.	Amaral Ferrador	S 30°51´34.5´´	W 52°15´02.5´´	1
Cunila menthoides Benth.	Canguçu	S 30°54´37.6´´	W 52°14´41´´	1
Cunila menthoides Benth.	Caçapava do Sul	S 30°32´28.2´´	W 53°33´18.7´´	1
Cunila platyphylla Epling	Vacaria	S 28°30´44´´*	W 50°56´02´´*	3
Cunila fasciculata Benth.	ICN 35910			1
Cunila incana Benth.	Caçapava do Sul	S 30°30´44´´ *	W 53°29´29´´*	3
Mentha arvensis L.	UCS-Caxias do Sul (Cultivated)	S 29°10´05´´*	W 51°10´46´´*	1
Rosmarinus officinalis L.	UCS-Caxias do Sul (Cultivated)	S 29°10´05´´*	W 51°10´46´´*	1

* Coordinates of nearby cities.

Table 2. ISSR primers used, number of polymorphic bands and Simpson Index

Primers	Nº of bands	Polymorphic bands (%)	Simpson's Index
$(CA)_8G$	09	100	0.877
$(CTC)_4RC$	21	100	0.921
$(AC)_8T$	24	100	0.883
$(AG)_8A$	11	72	0.596
$(GA)_8T$	14	92	0.864
$(AG)_8YC$	12	91	0.840
$(GACA)_4$	16	100	0.880
Total:	107	Average:	0.853

Table 3. Dice genetic similarities estimates among *Cunila* species computed from ISSR amplicon profiles. Each species are represented by the composed data of three samples, except for *C. fasciculata* , *C. origanoides* and the outgroups *M. arvensis* and *R. officinalis.*

	C. galioides	*C. spicata*	*C. microcephala*	*C. menthiformis*	*C. menthoides*	*C. platyphylla*	*C. fasciculata*	*C. incisa*	*C. angustifolia*	*C. incana*	*C. origanoides*	*M. arvensis*	*R. officinalis*
C. galioides	**0.964**	0.531	0.484	0.472	0.417	0.419	0.531	0.415	0.432	0.448	0.345	0.305	0.264
C. spicata		**1.000**	0.497	0.440	0.376	0.453	0.600	0.542	0.503	0.415	0.281	0.280	0.273
C. microcephala			**0.985**	0.788	0.516	0.543	0.597	0.528	0.396	0.469	0.400	0.387	0.316
C. menthiformis				**0.993**	0.527	0.460	0.493	0.507	0.421	0.478	0.447	0.341	0.341
C. menthoides					**0.995**	0.632	0.523	0.400	0.372	0.480	0.410	0.441	0.313
C. platyphylla						**1.000**	0.528	0.401	0.413	0.421	0.400	0.246	0.444
C. fasciculata							**1.000**	0.350	0.393	0.482	0.386	0.360	0.214
C. incisa								**0.992**	0.685	0.682	0.380	0.358	0.252
C. angustifolia									**0.993**	0.593	0.254	0.283	0.236
C. incana										**0.984**	0.400	0.317	0.214
C. origanoides											**1.000**	0.358	0.262
M. arvensis												**1.000**	0.444
R. officinalis													**1.000**

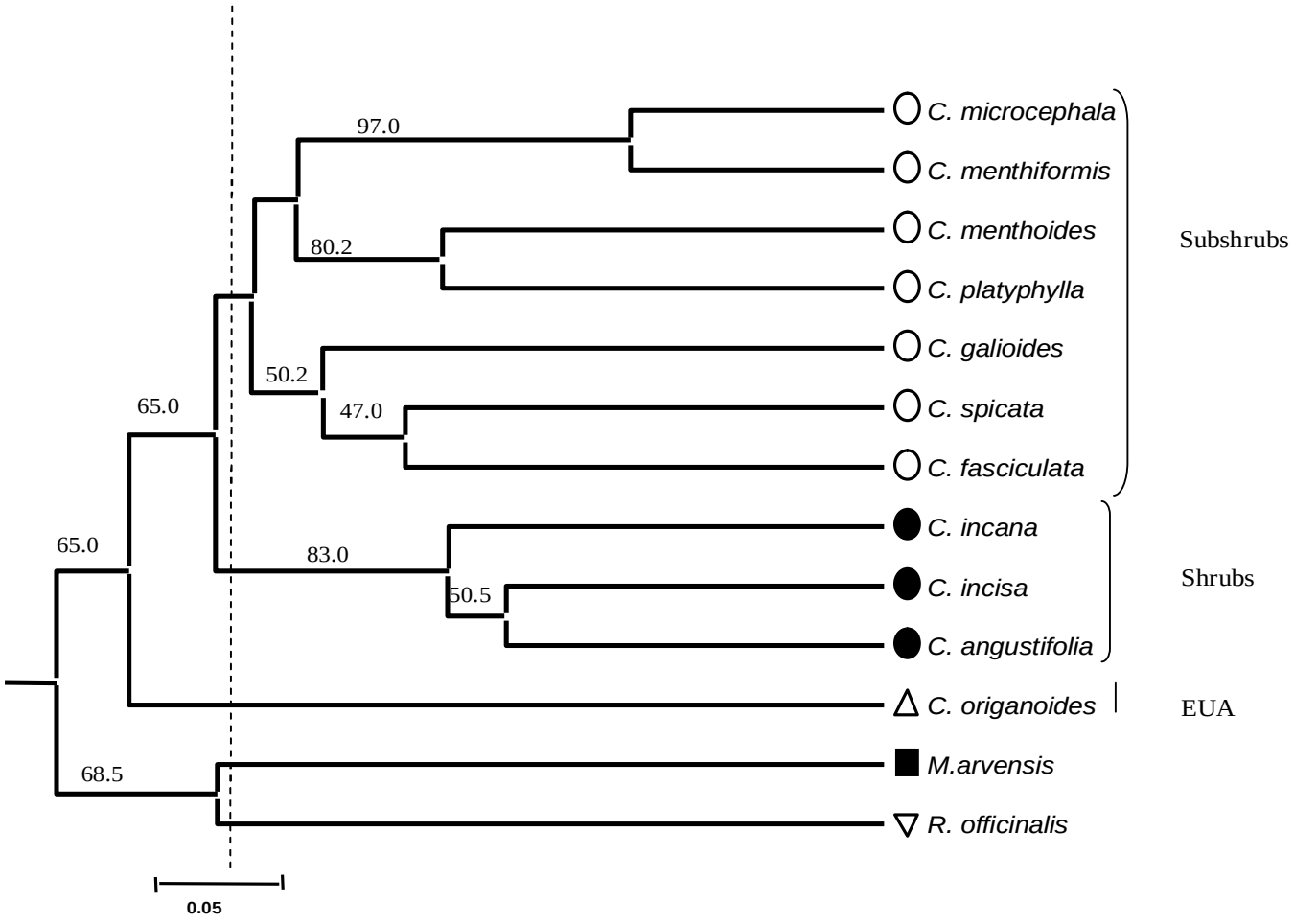

Figure 1. UPGMA dendrogram of genetic similarity (Dice coefficient) among *Cunila* species as constructed on the basis of ISSR markers. ○ Subshrubs section (*Spicatae* section); ● shrubs section (*Incanae* and *Incisae* sections); North American species Δ *C. origanoides* and outgroups ■ *M. arvensis* and ∇ *R. officinalis*. The bootstrap values are shown on the branches (1000 permutations).

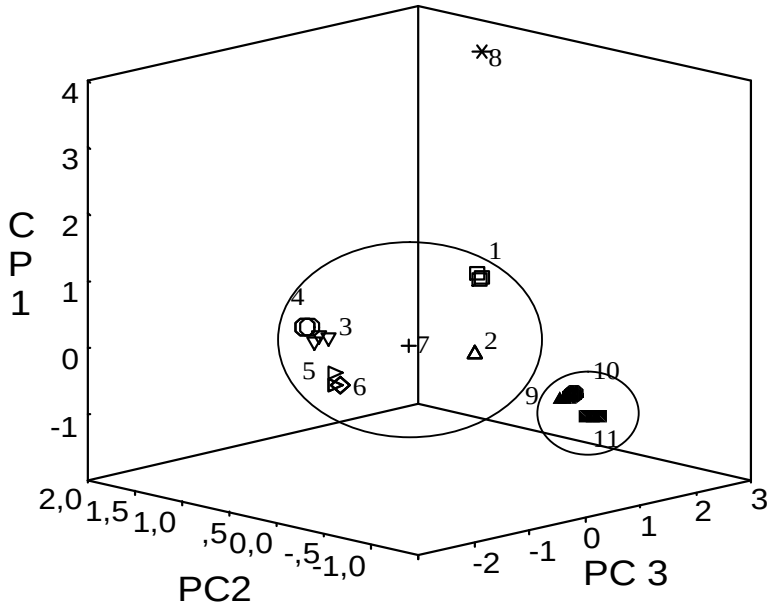

Figure 2. Principal component analysis of the 29 accessions of *Cunila* examined by ISSR markers. 1- *Cunila galioides* □, 2- *C. spicata* Δ, 3- *C. microcephala* ∇, 4- *C. menthiformis* ○, 5- *C. menthoides* ▷, 6- *C. platyphylla* ◇, 7- *C. fasciculata* ┼, 8- *C. origanoides* ∗, 9- *C. angustifolia* ▲, 10- *C. incisa* ●, 11- *C. incana* ■.

CAPÍTULO III

Variabilidade química e genética de *Cunila menthoides* Benth.

Inter and intrapopulational genetic variability inferred from ISSR data of Rio Grande do Sul (Brazil) populations of *Cunila menthoides* Benth.

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Abstract: *Cunila menthoides* are commonly used in South Brazilian folk medicine. Based on their prospective utility as aromatic and medicinal plants, the aim of this work was to examine the genetic variation within and among populations of *C. menthoides*. In this context, six *C. menthoides* populations collected in Rio Grande do Sul – Brazil, were analyzed by ISSR using ten primers that generated a total of 65 bands, of which 55.4% were polymorphic. Genetic distances were calculated using Jaccard´s coefficient, and cluster analysis was carried out using the UPGMA method. The populations were separated in four clusters which were in accordance with the geographic distribution. The six *C. menthoides* populations analyzed were genetically structured with low genetic variability, indicating that each population derives from a limited number of plants with low gene flow between populations.

Key words: *Cunila menthoides*; ISSR; genetic variability; inter and intrapopulations similarity.

Introduction

The genus *Cunila* D. Royen ex L. belongs to the Lamiaceae family, subfamily Nepetoideae, and tribe Mentheae (Cantino 1992). This genus presents two centers of distribution, one in North America and another in the southern of South America (Garcia-Peña 1989; Bordignon, 1997).

Several species of the genus *Cunila* are used in brazilian folk medicine, as stimulant, aromatic, antispasmodic, anti-inflammatory, emenagogue, antihelmintic, and in the treatment of chronic cough and respiratory infections (Simões et al., 1994). Moreover, *Cunila* essential oils have antibacterial, antifungal and insecticidal activity (Duke 1994; Luz et al., 2006) and recently have been tested against foodborne pathogens (Sandri et al., 2006). The *Cunila* species essential oil composition reveals a large variation (Bordignon et al., 1996, 1997, 1998, 1999).

The essential oils of *C. menthoides* Benth. are characterized by a large content of the monoterpens isomenthone (88.8%), menthone (4.7%) and pulegone (1.8%) (Bordignon et al., 1998), conferring to this species, anticonvulsive, sedative and analgesic principles, beyond preventive effect of cancer, among others (Guenther, 1975; Charalambous, 1994). Based on these chemical characteristics, this plant has the potential for the production of essential oils both for cosmetic and pharmaceutical purposes.

Cunila menthoides is characterized as a xilopodiferous subshrub with 20-50 cm with erect branches and ovate leaves. Flowering plants occur from September to November. This species is popularly known as “poejo-da-folha-grande” (big leaves poejo) being utilized as infusion for treatment of digestive upset, growing wild predominantly in dry and stone fields, being not abundant or common (Bordignon et al., 1998).

Despite of their economic value, species of *Cunila* have been poorly studied genetically among all genera of Lamiaceae.

Molecular markers have been developed during the last two decades and have largely overcome some problems associated with phenotype-based characterization of several plants. Firstly, isozymes and Restriction Fragment Length Polymorphisms (RFLP) were used as reliable markers for genetic analyses in plants (Botstein et al. 1980). More recently, PCR based techniques such as Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz et al., 1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos et al, 1995), provided a more rapid, easy to handle and cheaper approaches.

Among these, RAPD has been widely used to evaluate intraspecific variations in several aromatic and medicinal species of Lamiaceae, as *Salvia fruticosa* Mill. (Skoula et al. 1999), *Thymus vulgaris* L. (Echeverrigaray et al. 2001), *Cunila galioides* Benth. (Fracaro, et al. 2005) and *Cunila incisa* Benth. (Agostini, 2003). However, this method presents reproducibility problems that limit its use. Conversely, ISSR markers use longer primers that ensure higher reproducibility (Bornet and Branchard 2001; Reddy et al. 2002), and have a high capacity to reveal polymorphism offering a great potential to determine intra and interspecific levels of variation (Zietkicwicz et al. 1994, Shi et al. 2006). Within the family Lamiaceae, ISSR banding patterns were used to examine genetic diversity within and among populations of *Monarda fistulosa* L. var. *brevis* Fosberg & Artz (Kimball et al. 2001), *Hesperozygis ringens* (Benth.) Epling (Fracaro and Echeverrigaray, 2006), *Cunila spicata* Benth. (Albuquerque 2004) and *Lamiophlomis rotata* Benth. ex J. D. Hooker (Liu et al. 2006) and used to evaluate the genetic relationships among South American species of *Cunila* (Agostini et al., Cap. II).

Based on the aromatic and medicinal potential of this genus, the objective of the present work was to study the genetic diversity within and among populations of *C. menthoides.*

Material and methods

Plant material

Six populations of *Cunila menthoides,* with 10 samples plants from each population, were collected at different locations in Rio Grande do Sul State (Brazil) (Table 1; Figure 1) between October 2006 and March 2007. The analyzed populations were located at Camaquã, Caçapava do Sul, Amaral Ferrador, Barros Cassal, Vacaria and Canguçú.

DNA extraction and PCR amplification

Total genomic DNA was extracted by the CTAB method described by Doyle and Doyle (1987). Equal amounts (0.1g dry weight) of leaf tissue from each sample were placed in porcelain mortars refrigerated with liquid nitrogen and were ground with pestle to a fine powder. DNA samples were quantified in spectrophotometer at 260nm.

PCR amplifications were performed in a total reaction volume of 25 μl containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 0.2 mM each dNTP, 1.15 mM $MgCl_2$, 0.1 μM of primer, 1.5 unit of Taq polymerase (Cenbiot), 2% of DMSO and 50 ng of genomic DNA. DNA amplifications were performed using a thermal cycler TONAGEN PALM. The amplification conditions were one initial 5 minutes step at 92°C followed by 35 cycles of 94°C (1 min), 45s for annealing temperature

(48°C/50°C), and 72°C (2 min), the reactions were completed by a final extension step of 5 min (72°C) and 5 min (4°C).

The ISSR amplification products were stained by Syber Green (0.5 mg/mL - Invitrogen) and separated by horizontal electrophoresis in 1.5% agarose gel soaking in 1X TBE buffer (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) at 70mA.

Amplifications products were visualized and photographed under ultra violet light. The size of the amplicons was determined by comparison with 100 bp Ladder molecular weight (CENBIOT).

Nine anchored oligonucleotide primers $(CTC)_4RC$, $(AG)_8A$, $(AG)_8YC$, $(GA)_8YC$, $(GA)_8T$, $(AC)_8T$, $(CA)_8G$, $(GT)_8A$, $(GT)_8T$, and one nonanchored primer, $(GACA)_4$, were used to amplify all samples. These primers were selected from a set of 20 ISSR primers based on the number of amplification products and the quality of the profiles obtained with one sample of each population.

Statistical analyses

Bands were scored as a binary variable, (1) for presence and (0) for absence of each amplicon. The binary matrix (1/0) was used to calculate the similarity by Jaccard coefficient among each pair of samples. The relationship among species was evaluated constructing dendrograms by UPGMA (Unweighted Pair Group Method Using Arithmetic Averages). These statistical analyses were performed using NTSYS Package (Rohlf, 2001) and the SPSS 10.0 software (SPSS Inc., Chicago, Illinois). The permutation analysis (1000 permutations) was performed using the WinBoot (Yap and Nelson 1996) and the analysis AMOVA was conducted by GenAlEx (Peakall and Smouse 2001) program. The program STRUCTURE version 2.2 (Pritchard et al., 2000)

was used to identify distinct genetic populations, assigning individuals to populations and identify migrants and admixed individuals.

Results and discussion

According to Bordignon (1997) and Coelho de Souza (1997), *Cunila menthoides* is limited to a few regions of Rio Grande do Sul – Brazil, being collected in just seven points in the state. It is important to mention that despite our efforts to collect a high number of populations, just six were found, being three of these, collected in localities not previous reported.

To test the potential of each ISSR primer, 20 primers were screened against total DNA from one sample of each population. Based on the number of amplification products, the quality of the profiles, the level of polymorphism and the reproducibility, ten primers were selected for further studies of *C. menthoides*. The use of few, but highly informative primers, lowers the cost, time and labors for diversity analysis (Reddy et al, 2002), this practice is reported in other genera (Charters et al., 1996; Lanham and Brennan, 1998; Gilbert et al., 1999; Prevost and Wilkinson, 1999).

Applied to the 60 samples under study, the ten selected primers generated a total of 65 amplified fragments, 36 of which were polymorphic (55.4%) (Table 2). The number of amplified products per primer varied from four ($(AG)_8A$ and $(CA)_8G$) to 11 ($(CTC)_4RC$). The mean number of segments amplified by a primer was 6.5 and the size of the amplified products ranged from 150 to 1600 bp.

The analyses revealed populations-specific markers for all populations analyzed. For instance, primer $(AG)_8A$ revealed a fragment of 1400bp that characterized the population of Canguçu, primers $(GT)_8A$ and $(GA)_8YC$ revealed fragments of 800bp and

500bp, respectively, that were characteristics of Vacaria samples. The Camaquã population, was characterized by the presence of amplicons with 580bp and 250bp with primer $(GT)_8T$. At last, primer $(CTC)_4RC$ reveled two specific bands for Amaral Ferrador (800bp and 750bp) and one for Caçapava populations (200bp). Species specific markers revealed by ISSR have been documented in the genus *Cunila* Royen ex L. (Agostini et al., 2008 CapII) as well in other genera, like *Vigna* (L.) Hepper (Ajibade et al. 2000), *Cicer* L. (Sudupak 2004), *Lycoris* Herb. (Shi et al. 2006) and *Stachys* L. (Kochieva et al. 2006).

The Jaccard similarity calculated based on the ISSR markers for each pair of populations varied from 0,704 to 0,910. The highest similarity values between populations were obtained for the comparison between Amaral Ferrador and Camaquã (0.910), both populations belonging to the "Encantada" mountains and separate by 76Km. The lowest similarity values were obtained between Barros Cassal and Camaquã (0.704). The mean similarity calculated by AMOVA within populations was very high (93%), indicating that the populations analyzed are distinct genetic pools. This high similarity may be associated with restrict genetic bases reflecting founder effect. Probably each population was established by a very small number of individuals with low genes flow among populations, or the populations passed through a "bottleneck" in which only a few individuals survive, and later expands again under more favorable conditions.

Cluster analysis based on genetic similarities values (Jaccard's coefficient) was conducted to generate a dendrogram indicating relationships among the populations of *C. menthoides*. Cluster analysis (Figure 2) showed the populations splitted into four groups with high bootstrap values, according with the geographical distribution of the populations. Group 1 was formed by population of Vacaria, situated in the grasslands of

high altitudes of the Atlantic range. The second group was formed by two subgroups, one including population belonging to Camaquã and other corresponding to Amaral Ferrador, both populations belonging to the "Encantada" mountains. Group 3 was composed by the population representing Canguçu, belonging to the Rio Grande do Sul Serra do Sudeste. The populations of Camaquã, Amaral Ferrador and Canguçu are very close, but Canguçu are separated from the others by the Camaquã River and possible because of this, the genes flows are reduced. The fourth group was formed by two subgroups, the first includes population of Caçapava do Sul located in the central region of the state and the second includes the populations of Barros Cassal belonging to the Encosta Inferior do Nordeste. The separation of these groups among *C. menthoides* populations was confirmed by analyses of molecular variance (AMOVA), principal components (Figure 3) and STRUCTURE analysis (data not shown), based on these data, we can infer that all the populations analyzed form distinct genetic pools, with no migrants and admixed individuals.

All populations analyzed in this work showed limited number of individuals and were found in fragmented environments threatened by local agriculture and pasture characteristics of the region. The population of Vacaria showed the lowest number of individuals, being the population less vigorous. Vacaria are characterized by a high cattle activities and strong agriculture of apple, maize and soybean cultures among others.

The limited number of individuals may lead to increased homozygosity with reduction of the vigor of individuals, expression of deleterious characters, increased seed abortion, and reduced fertilization and germination rates, leading consequently to the disappearance of the population (Dubash and Fenster, 2000). In addition, in fragmented environments may exist a tendency to lose intrapopulation genetic

variability due to the low frequency of interpopulational gene exchange (Oostermeijer et al., 2003). The combination of these facts indicate the need to maintain small-scale protected areas, as indicated by Laguna et al. (1998), for endemic, rare or endangered plants.

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Table 1- Geographical coordinates of the six *Cunila menthoides* Benth. populations collected in Rio Grande do Sul-Brazil.

Population	Latitude	Longitude	N° of samples
Vacaria	S 28° 30' 44"	W 50° 56' 02"	10
Barros Cassal	S 29°06´18.4´´	W 52°40´13.8´´	10
Caçapava do Sul	S 30°32´28.2´´	W 53°33´18.7´´	10
Amaral Ferrador	S 30°51´34.5´´	W 52°15´02.5´´	10
Camaquã	S 30° 51' 04"	W 51° 48' 44"	10
Cangucú	S 30°54´37.6´´	W 52°14´41´´	10

Table 2- ISSR primers used number of bands and polymorphic bands.

Primers	Nº of bands	Polymorphic bands (%)
$(CTC)_4RC$	11	7
$(AG)_8A$	4	3
$(AG)_8YC$	5	3
$(GA)_8YC$	6	2
$(GA)_8T$	5	2
$(AC)_8T$	6	2
$(CA)_8G$	4	1
$(GT)_8A$	7	5
$(GT)_8T$	8	5
$(GACA)_4$	9	6
Total	65	36

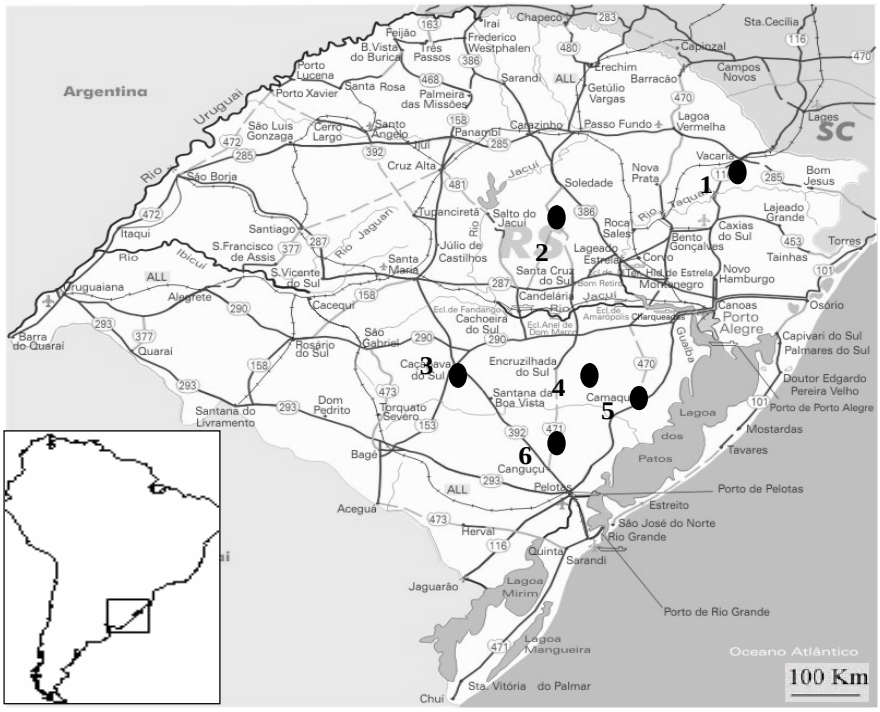

Figure 1- The geographical origin of the six populations of *Cunila menthoides* Benth. collected in Rio Grande do Sul-Brazil. 1-Vacaria; 2-Barros Cassal; 3-Caçapava do Sul; 4-Amaral Ferrador; 5-Camaquã; 6-Canguçu.

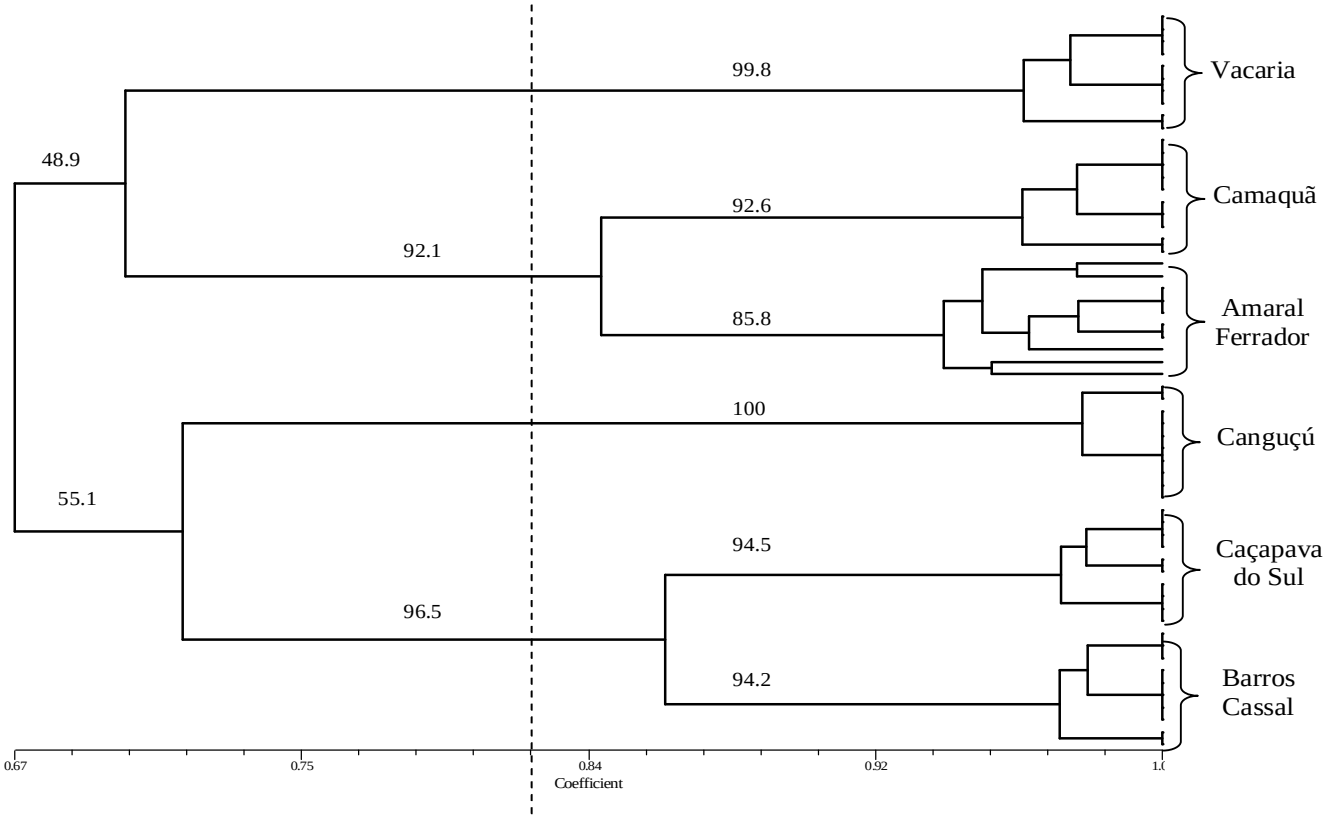

Figure 2- UPGMA dendrogram of genetic similarity (Jaccard´s coefficient) among *Cunila menthoides* Benth. populations constructed on the basis of ISSR markers. The values on the branches correspond to Bootstrap (1000 permutations).

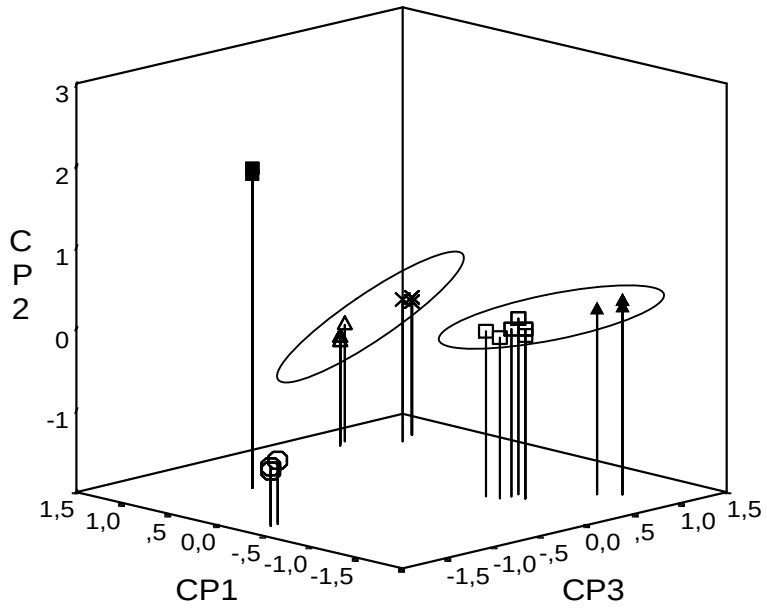

Figure 3- Principal component analysis of the six populations (60 individuals) of *Cunila menthoides* examined by ISSR markers. ○ Vacaria; ▲ Camaquã; □ Amaral Ferrador; ■ Canguçu; x Caçapava do Sul and Δ Barros Cassal.

Chemical variation in the essential oil composition of *Cunila menthoides* Benth. (Lamiaceae).

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Abstract: *Cunila menthoides* is characterized as a xilopodiferous subshrub with strong and pleasant smell. This species are commonly used in South Brazilian folk medicine. Air-dried samples of individual plants of four Brazilian populations of *C. menthoides* were extracted by steam distillation and analyzed using GS and GS–MS. A total of 15 volatile compounds were detected and identified. Two groups, which can be considered as chemotypes, were formed by the average linkage cluster analysis. The first group was characterized by high concentrations of pulegone, with some individuals showing variations to menthone and the second group characterized by high concentrations of linalool.

Key words: *Cunila menthoides*; aromatic and medicinal plants; menthone; pulegone; linalool; chemotype.

Introduction

The genus Cunila D. Royen ex L., family Lamiaceae, subfamily Nepetoideae, tribe Mentheae (Cantino et al., 1992) consists in approximated 20 species with two centers of distribution: North America with nine species and the southern part of South America with eleven species (Epling, 1936; Garcia-Peña, 1989; Bordignon, 1997). The South American species are divided into three sections: *Incana*, *Incisae* and *Spicatae* (Epling, 1936). *Cunila menthoides* Benth. belong to the section *Spicatae*, characterized by subshrubs or evergreen perennial herbs with terminal spikes or globular inflorescences formed by small almost glabrous flowers (Epling, 1936).

Cunila menthoides grows wild in southern Brazil and Uruguay, predominantly in dry and stony fields. This species is characterized as a xilopodiferous subshrub with 20-50 cm with erect branches. The leaves are ovate, and flowers and fruits are found from September to November. This species is popularly kwon as "poejo-da-folha-grande" (big leaves poejo) being utilized as infusion for the treatment of digestive upsets (Bordignon et al., 1998).

The essential oil of *C. menthoides* is characterized by a large content of the monoterpens isomenthone (88.8%), menthone (4.7%) and pulegone (1.8%), conferring to this specie, anticonvulsive, sedative and analgesic principles, beyond preventive effect of cancer, among others (Bordignon, 1997). Based on these facts, this plant has potential for the economic production of essential oils both for cosmetic and pharmaceutical purposes.

References to the oil composition of South American species of the genus *Cunila* reveal a large variation (Manns and Hartmann, 1992; Bordignon et al., 1996, 1997, 1998, 1999; Echeverrigaray et al., 2003; Agostini et al., 2006; Echeverrigaray et al., 2008), meanwhile, intra-specific variations in the chemical composition were

observed in *C. angustifolia* Benth. (Bordignon et al., 1999; Moreira and Krambeck, 1976), *Cunila galioides* Benth. (Echeverrigaray et al., 2003) and more recently in *Cunila spicata* Benth. (Echeverrigaray et al., 2008).

Terpenes are among the most widespread and chemically diverse groups of compounds produced by plants. Several biological activities in plants have been atributed to terpenes, including: hormonal, mediators of polysaccharide synthesis, photosynthetic pigments, electron carriers, and membrane components, among others (Chappell 1995; McGarvey and Croteau 1995). Terpenes also mediate interactions between plants and their environment producing toxic defense compounds to ward off herbivores or fungal attacks (Johnson and Croteau 1987; Lewinsohn et al., 1992), or producing volatile compounds released from the plants to attract pollinators (Dobson, 1993).

The use of secondary metabolites in plant taxonomy is well recognized (Gottlieb, 1982), as these compounds can sometimes aid in taxonomical classification. Moreover, an essential oil analysis has been used with success on the study of intra-specific diversity and geographic patterns of variation in several plant species, including several representatives of the Lamiaceae family: *Thymus* sp. L. (Cañigueral et al., 1994), *Salvia fruticosa* Mill. (Skoula et al., 1999), *Ocimum gratissimum* L. (Vieira et al., 2001), *Cunila incisa* Benth. (Agostini et al., 2006), *Cunila galioides* Benth. (Echeverrigaray et al., 2003) and *Cunila spicata* Benth. (Echeverrigaray et al., 2008) among other.

In the present work, we report the chemical (essential oil composition) relationship within and among four populations of *C. menthoides*, collected at different locations all over the distribution area of this species in South Brazil.

Material and methods

Plant material

Cunila menthoides flowering plant samples were collected at four locations in Rio Grande do Sul State, Brazil (Table 1), between October 2006 and March 2007. The populations analyzed were: Caçapava do Sul; Amaral Ferrador; Barros Cassal and Canguçú, each population composed by five individuals. Populations belonging to Vacaria and Camaquã were found but cannot be used in these experiments because of the poor volume of individuals.

The individual plant samples of each population were conditioned in plastic bags and transported to the laboratory under refrigeration. Samples (~200 g) were air-dried at room temperature (20–25 °C), and maintained in a refrigerated chamber (10 °C) until extraction.

Volatile oil analysis

The air-dried samples (50 g) were subjected to distillation for 1 h using a Clevenger-type apparatus. The oil obtained was separated from water and dried over anhydrous Na_2SO_4.

Gas chromatographic (GC) analysis were performed with a Hewlett Packard 6890 Series equipped with a HP-Chemstation data processor, fitted with a HP-Innowax bonded phase capillary column (30 m x 0.32 mm i.d., 0.50 μm film thickness, Hewlett Packard, Palo Alto, USA); column temperature, 40°C (8 min) to 180°C at 3°C/min, 180º–230°C at 20°C/min, 230°C (20 min); injector temperature 250°C; detector temperature 250°C ; split ratio 1:50; carrier gas H_2 (34KPa). Volume injected 1 μL diluted in hexane (1:10).

Gas chromatograph coupled with a mass selective detector (GC/MS) analysis were performed in a HP 6890 GC using a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. A fused silica capillary column HP-Innowax (30 m x 0.25 mm), 0.25μm film thickness (Hewlett Packard, Palo Alto , USA) was used. The temperature program was the same used in the gas chromatography (GC) analysis: interface 280°C; split ratio 1:100; carrier gas He (56 KPa); flow rate: 1.0 mL/min; ionization energy 70 eV; mass range 40-350; volume injected 0.4μL diluted in hexane (1:10). Identification of the individual components was based on comparison of their GC retention indices (RI) on polar columns and comparison with mass spectra of components by GC/MS.

Statistical analysis

All the constituents identified in the essential oils were used to calculate Mean Euclidian distances. To avoid the dependency of percentage data, peak areas were standardized within sample by dividing the peak area of each variable (component) by the average of all components of the sample. Cluster analysis, dendrograms, canonical discriminant analysis and principal component analysis were performed by SPSS (version 10.1).

Results and discussion

Analyses of the essential oil composition of the four populations of *C. menthoides* (20 samples), extracted by steam distillation and analyzed by GC-MS, identified 15 compounds (Table 2), including oxygenated and non-oxygenated forms of monoterpenes and sesquiterpenes, that represented between 69.26% and 96.64% of the total essential oil extracted.

The dendrogram (Fig. 1) represents graphically the relationship between the populations and the similarity groups, based on their essential oil composition. Two groups were formed by the Average Linkage cluster analysis: the first was formed by the populations from Canguçu, Caçapava do Sul and Amaral Ferrador, this group was divided into two subgroups corresponding to the samples with high concentration of pulegone, and the samples showing a variation in the percentage of menthone/pulegone. The second group was formed by the population from Barros Cassal and is representative of a new chemotype of *C. menthoides*, characterized by a high content of linalool.

As previously reported by Bordignon et al. (1998), the main constituent in the essential oil of *C. menthoides*, collected in Amaral Ferrador, is isomenthone (88.8%), menthone (4.7%) and pulegone (1.8%). Differing from this data, this work indicates the existence of two chemotypes: pulegone and linalool (Figure 2). Plants with high concentration of isomenthone were not reported in this work, the concentrations of isomenthone ranged from 0 to 2.01±0.47, in plants from Barros Cassal and Caçapava do Sul, respectively. The high concentrations of isomenthone reported previously (Bordignon et al., 1998) can indicate the existence of a third chemotype. Other species belonging to the genus *Cunila* showed chemotypes, this is the case of *C. angustifolia* (Moreira and Krambeck, 1976; Bordignon et al., 1999), *C. galioides* (Echeverrigaray et al., 2003) and *C. spicata* (Echeverrigaray et al., 2008). The other population studies involving *C. incisa* species doesn't showed qualitative variations in the essential oil main compound (Bordignon et al., 1996; Agostini et al., 2006).

The samples representing Caçapava do Sul population were divided in the dendrogram (Figure 1) based in the essential oil main compounds. Two samples showed pulegone as principal compound (51.25±1.02) and three samples were characterized by

menthone (53.41±4.91). Differences in the concentrations of these compounds were observed among the individuals corresponding to this population. These differences ranged from 38.7% (sample Caçapava 4) to 57.5% (sample Caçapava 2) for menthone, and 28.8% to 51.9%, samples Caçapava 2 and Caçapava 4, respectively, for pulegone.

Pulegone are the menthone precursor in the monoterpene biosynthetic pathway. Thus, after the conversion of the primary metabolites isopentenyl diphosphate and dimethylallyl diphosphate to geranyl diphosphate (GPP), the cyclization of this universal monoterpene precursor to the committed intermediate (-)-limonene, and the cytochrome P450-mediated hydroxylation to (-)-transisopiperitenol, a sequence of five steps produce (-)-menthol. In this context, the monoterpene ketone (+)-pulegone assumes central importance because it is the precursor of (-)-menthone, (-)-menthol, and of the sideproduct (+)-menthofuran (Bertea et al., 2001). Depending on environmental or genetic conditions, this branch point metabolite may be reduced to (-)-menthone in route to menthol, by pulegone reductase (PR) (Mahmoud e Croteau, 2003). In the case of *C. menthoides*, overexpression and cosuppression of PR can result in the respective increase or decrease in the production of menthone and pulegone, the reduction of the subsequent product accompanies the accumulation of the intermediate. The variation observed between the percentage of pulegone and menthone, in Caçapava do Sul population, can indicate a variation in the activity of pulegone reductase. More chemical analyses involving a larger number of plants from this population, and in different development stages are required to confirm the existence of two chemotypes, pulegone and menthone, or confirm the hypothesis of variations in enzyme PR actions in some individuals.

Other chemotype observed in *C. menthoides* showed high concentrations of linalool. This compound is derived from isopentenyl pyrophosphate via the universal

isoprenoid intermediate, geranyl pyrophosphate, which is used as substrate by linalool synthase (LIS) (Cseke et al., 1998; Raguso and Pichersky, 1999). Linalool is an acyclic monoterpene alcohol with pleasant fragrance that occurs among diverse monocot and eudicot families (Knudsen et al., 1993). This compound is prized by the flavor and fragrance industry as a component of bergamot and lavender essential oils and numerous commercial perfumes (Hanneguelle et al., 1992; Ohloff, 1994).

The pulegone chemotype shared just three compounds with linalool chemotype: sabinene, mircene and limonene. Sabinene was detected in high concentration in the linalool chemotype (13.65+6.72), but in low concentrations in the other plants (from 0.31±0.06 to 0.40±0.03). (+)-cis-sabinene hydrate and (+)-trans-sabinene hydrate are the main monoterpenes found in marjoram (*Origanum majorana*), but can also be found in other *Origanum* species. The synthesis of sabinene hydrate in marjoram (*Origanum majorana*) is performed by sabinene hydrate synthase from the precursor GPP (Novak et al., 2000). Mircene and limonene appeared in low concentration for all populations analyzed. Mircene ranged from 0.15±0.22 in Barros Cassal population to 0.47±0.13 in Caçapava do Sul (B) population, and limonene ranged from 2.94±1.45 in Barros Cassal population to 5.32±1.08 in Amaral Ferrador population.

Chemical variation between populations can be attributed to genetic and/or environmental factors (Hay and Waterman, 1993). However, as the plants examined were cultivated under the same conditions, the variations observed may be under genetic rather than environmental control. The variation of essential oil composition has ecological advantages protecting plants against pathogens or climatic conditions, and favoring pollination. Moreover, the chemical variation observed indicates that selection, breeding and croping of specific chemotypes are necessary for the proper commercial use of *C. menthoides*.

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Table 1- Geographical coordinates of the four *Cunila menthoides* Benth. populations collected in Rio Grande do Sul-Brazil.

Population	Latitude	Longitude	N° of samples
Barros Cassal	S 29°06´18.4´´	W 52°40´13.8´´	5
Caçapava do Sul	S 30°32´28.2´´	W 53°33´18.7´´	5
Amaral Ferrador	S 30°51´34.5´´	W 52°15´02.5´´	5
Canguçú	S 30°54´37.6´´	W 52°14´41´´	5

Table 2- Chemical composition (relative percentage of total volatile oils) of *Cunila menthoides* Benth. populations collected in Rio Grande do Sul-Brazil.

	RT	**Canguçu**	**Caçapava do Sul (A)**	**Caçapava do Sul (B)**	**Amaral Ferrador**	**Barros Cassal**
α-pinene	6.336	0.56±0.18	0.52±0.21	0.69±0.01	0.63±0.09	0
β-pinene	10.569	0.6 ±0.17	0.64±0.17	0.78±0.02	0.77±0.14	0
sabinene	11.448	0.36±0.09	0.31±0.06	0.40±0.03	0.39±0.07	13.65±6.72
α-tujene	12.841	0	0	0	0	0.18±0.18
mircene	14.083	0.17±0.02	0.45±0.01	0.47±0.13	0.27±0.07	0.15±0.22
limonene	15.582	3.40±1.29	2.71±0.03	4.62±1.29	5.32±1.08	2.94±1.45
α-terpinene	21.905	0	0	0	0	0.57±1.16
γ-terpinene	23.754	0	0	0	0	0.9±0.3
trans-β-ocimene	24.554	0	0	0	0	1.86±1.52
isomenthone	27.762	1.24±0.19	1.5±0.01	2.34±0.13	1.33±0.09	0
menthone	28.976	28.31±4.48	39.31±0.85	53.41±4.91	30.16±2.44	0
pulegone	35.091	60.57±3.76	51.25±1.02	33.2±6.57	57.74±0.75	0
linalol	37.106	0	0	0	0	40.94±12.33
β-cariophylene	39.225	0	0	0	0	2.56±1.05
bicyclegermacrene	44.273	0	0	0	0	4.47±2.38
caryophyllene oxide	52.628	0	0	0	0	0.99±0.34

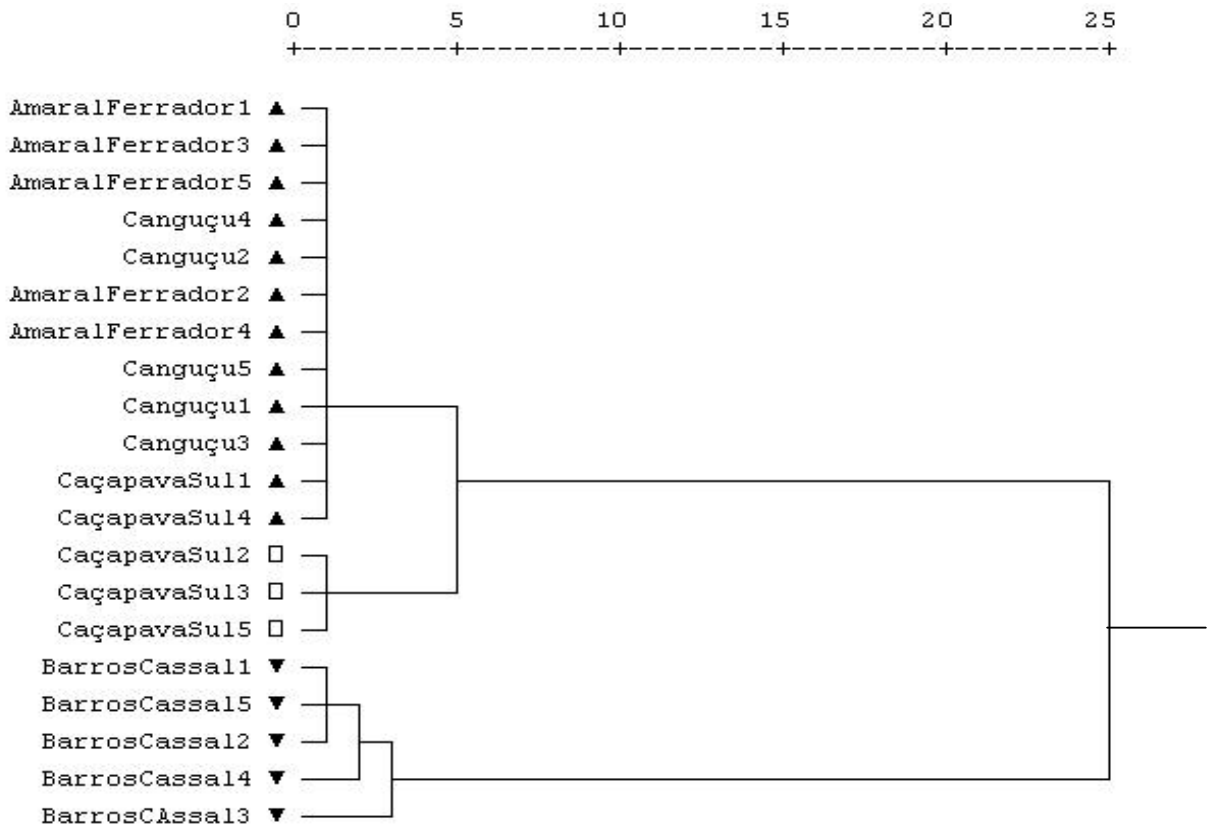

Figure 1- Dendrogram using Average Linkage (Between Groups), constructed based on the essential oil composition of four populations of *Cunila menthoides* Benth., collected in Rio Grande do Sul-Brazil. Pulegone chemotype ▲; menthone/pulegone □; linalool chemotype ▼.

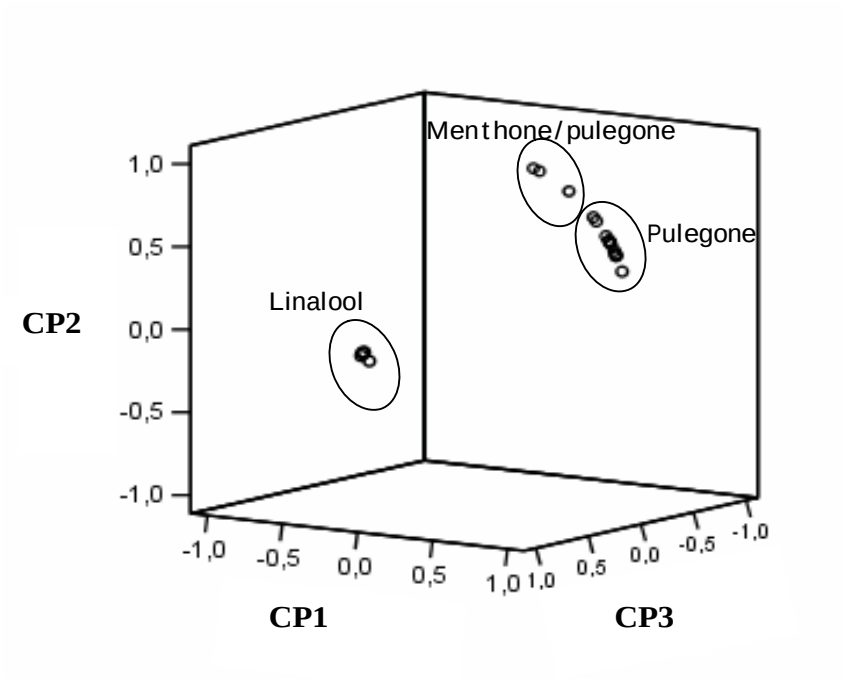

Figure 2- Principal component analysis based on the essential oil composition of four populations of *Cunila menthoides* Benth., collected in Rio Grande do Sul-Brazil. Pulegone chemotype corresponding to two samples of Caçapava do Sul and all samples of Amaral Ferrador and Canguçu; linalool chemotype represented by all samples of Barros Cassal and three samples of Caçapava do Sul corresponding to the menthone/pulegone group.

CAPÍTULO IV

Composição química do óleo essencial de *Cunila incana* Benth.

Artigo formatado para a revista "*Journal of Essential Oil Research*", no status de artigo submetido.

Essential oil composition of *Cunila incana* Benth. (Lamiaceae).

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Abstract

The essential oil obtained from *Cunila incana*, a wild plant from South Brazil, was analyzed by GC and GC/MS. The main volatile compounds were the sesquiterpenes β-caryophyllene (11,12% ± 0,15), palustrol (10,99% ± 0,81), germacrene D (10,99% ± 0,81), and the monoterpene *trans*-β-ocimene (10,36% ± 0,48). Differing from the essential oil obtained from the others *Cunila* species, *C. incana* essential oil showed low concentration of monoterpenes (20,66%) and high sesquiterpenes (59,94%) content.

Keywords

Cunila incana; Lamiaceae; essential oil composition; terpenes; *trans*-β-ocimene; β-caryophyllene; palustrol; germacrene.

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Introduction

The genus *Cunila* D. Royen ex L., belongs to the family Lamiaceae, subfamily Nepetoideae and tribe Mentheae (1). This genus consists in approximated 20 species with two centers of distribution, one in North America with nine species and other in the southern region of South America with 11 species (2, 3).

Several species of the genus *Cunila* are used in Brazilian popular medicine, as stimulant, aromatic, antispasmodic, emenagogue, antihelmintic, and in the treatment of chronic cough and respiratory infections (3). Moreover, *Cunila* essential oils have antibacterial, antifungal and insecticidal activity (4, 5) and recently have been tested against foodborne pathogens (6). The essential oil composition of South American species of the genus *Cunila* reveals a large variation with menthofuran, 1,8-cineole, isomenthone, menthone, pulegone, oxide *trans*-piperitone, sabinene, linalol, citral, β-ocymene, as the mains constituents depending on the species (7-13).

Based on growth habit and inflorescence morphology, the South American species were classified into three sections *Incanae*, *Incisae* and *Spicatae* (14).

Cunila incana Benth. belongs to the section *Incanae*, being the only species of this section. This species is characterized as a shrub with 1 to 2 m and differencing from the other sections mainly by the presence of single hairy pale yellow flowers at upper axils. The leaves are spatulate and oblanceolate, flowers and fruits are found from September to December. Despite their morphological differences, the shrub sections *Incanae* and *Incisae* are very similar genetically (15).This plant can be seen growing wild in southern Brazil, Argentina and Uruguay, predominantly in dry and stony fields (3).

In the present work, the chemical composition of the essential oil from *C. incana* is described for the first time.

Experimental

Plant material and extraction

Cunila incana flowering plant samples were collected at Caçapava do Sul, Rio Grande do Sul State, Brazil, in November 2007. Voucher specimens have been deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (Brazil).

The air-dried samples (50 g) were subjected to distillation for 1 h using a Clevenger-type apparatus. The oil obtained was separated from water and dried over anhydrous Na_2SO_4.

Gas chromatography

Gas chromatographic (GC) analysis were performed with a Hewlett Packard 6890 Series equipped with a HP-Chemstation data processor, fitted with a HP-Innowax bonded phase capillary column (30 m x 0.32 mm i.d., 0.50 μm film thickness, Hewlett Packard, Palo Alto, USA); column temperature, 40°C (8 min) to 180°C at 3°C/min, 180º–230°C at 20°C/min, 230°C (20 min); injector temperature 250°C; detector temperature 250°C ; split ratio 1:50; carrier gas H_2 (34KPa). Volume injected 1 μL diluted in hexane (1:10). Gas chromatograph coupled with a mass selective detector (GC/MS) analysis were performed in a HP 6890 GC using a mass selective detector Hewlett Packard MSD5973, equipped with HP Chemstation software and Wiley 275 spectra data. A fused silica capillary column HP-Innowax (30 m x 0.25 mm), 0.25μm film thickness (Hewlett Packard, Palo Alto , USA) was used. The temperature program was the same used in the gas chromatography (GC) analysis: interface 280°C; split ratio 1:100; carrier gas He (56 KPa); flow rate: 1.0 mL/min; ionization energy 70 eV; mass range 40-350; volume injected 0.4μL diluted in hexane (1:10). Identification of the

individual components was based on comparison of their GC retention indices (RI) on polar columns and comparison with mass spectra of components by GC/MS.

Results and Discussion

Hydrodistillation of the dried aerial parts of *C. incana*, at the flowering stage, yielded a mean of 0.72% (v/w). The average yield of other *Cunila* species ranged from 0.16% to 1.30% (7-13, 19). The results of the qualitative and quantitative analysis by GC/MS of the essential oil constituents are shown in Table 1 in order of elution from the HP Innowax column.

A total of 13 compounds corresponding to 80.6% of the essential oil were identified. These compounds included four monoterpenes (α-pinene, β-pinene, limonene, and trans-β-ocimene), and nine sesquiterpenes (α-gurjunene, β-cariofilene, β-elemene, D-germacrene, β-selinene, α-selinene, bicyclogermacrene, palustrol and ledene). The remaining 19.4% of the essential oils corresponded to traces or non identified compounds. Only one oxygenated terpene (palustrol) was identified.

Comparison of the essential oil composition of samples I and II allowed to detect a qualitative variation (presence or absence of α-pinene), but non evident quantitative differences for the other compounds. The main volatile compounds were the sesquiterpenes β-caryophyllene (11.12% $\pm$ 0.15), palustrol (10.99% $\pm$ 0.81), germacrene D (10.99% $\pm$ 0.81), and the monoterpene trans-β-ocimene (10.36% $\pm$ 0.48). Many constituents present in the essential oil of *C. incana* have insecticidal, herbicidal, spasmolytic and/or anti-inflammatory activities (16, 17), indicating a potential use of this oil.

Monoterpenes comprises the major components of the essential oils of most Lamiaceae species (18), including both South and North American *Cunila* species

(approximately 75-95%) (7, 9-13, 19-21). Conversely, *C. incana* essential oil showed low concentration of monoterpenes (20.66%) and high sesquiterpenes (59.94%) content.

Two distinct and independent terpenes biosynthetic routes have been described in plants: the cytosolic pathway to isopentenyl diphosphate (IPP) starts from acetyl-CoA and proceeds through the classical intermediate mevalonic acid to provide precursors for the biosynthesis of sesquiterpenes and triterpenes, and the plastidial pathway which initiate by the transketolase-type condensation of pyruvate and glyceraldehyde-3-phosphate to 1-deoxyxylulose-5-phosphate (DXP) providing precursors for the biosynthesis of isoprenes, monoterpenes, diterpenes and tetraterpenes (18). The monoterpene biosynthesis in mint is specifically localized to the glandular trichomes and originates in the leucoplasts of the secretory cells of these specialized nonphotosynthetic epidermal structures, but during the brief and intense period of secretory activity, monoterpene biosynthesis is driven by plastidial supply of IPP and its allylic isomer DMAPP (dimethylallyl diphosphate) via the DXP pathway (18, 22). Despite the presence of glandular trichomes, the high concentration of sesquiterpenes over monoterpenes in *C. incana* essential oil indicates that the mevalonic acid pathway is the main terpene biosynthetic route adopted by this species.

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Table 1: Compounds identified by GC/MS of the two analyzed samples of *Cunila incana* collected in Caçapava do Sul, R.S. – Brazil.

Compound	Sample I (%)	Sample II (%)
α-pinene	3,01	----
β-pinene	3,44	2,78
limonene	5,97	5,41
trans-β-ocimene	10,84	9,88
α-gurjunene	2,52	2,48
β-caryophyllene	11,27	10,96
β-elemene	10,09	9,38
germacrene-D	11,45	10,41
β-selinene	2,59	3,04
α-selinene	2,59	2,98
bicyclogermacrene	4,17	3,96
palustrol	10,17	11,80
ledene	3,85	6,15

CONSIDERAÇÕES FINAIS

A região sul do Brasil apresenta particularidades climáticas que a tornam peculiar, tal como clima temperado com períodos de temperaturas baixas, solos argilosos, alta pluviosidade, elevada umidade do ar, entre outras. Muitas espécies vegetais pertencentes aos campos da região sul, principalmente do Rio Grande do Sul, apresentam potencial quanto ao uso em industrias farmacêuticas, cosméticas, alimentícias, entre outras. Este é o caso particular das espécies do gênero *Cunila*.

Este gênero vem recebendo, nos últimos anos, atenção e dedicação científica, levando-se em consideração seu potencial como planta aromática e medicinal.

Os resultados obtidos neste trabalho, a partir do seqüenciamento de regiões nucleares e plastidiais, confirmam a eficiência destas regiões do DNA para análises filogenéticas ao nível genérico, como já observado por outros autores, os quais propuseram filogenias para outras espécies aromáticas e medicinais. A região nuclear ITS se mostrou mais informativa, enquanto que a região plastidial *trn*L-*trn*L-*trn*F apresentou-se mais conservada. Baseado nos resultados apoiados pelos tratamentos estatísticos apresentados aqui se pode observar o estado parafilético do gênero *Cunila*, o qual apresenta suas espécies em dois grupos principais correspondendo às espécies Norte e Sul-Americanas.

Dentre as espécies Sul Americanas, as três seções propostas por Epling (1936) não se apresentam geneticamente estáveis. Sugere-se assim, no presente trabalho, a união de duas seções geneticamente muito similares, as seções arbustivas *Incanae* e *Incisae*.

Paralelamente ao estudo filogenético do gênero *Cunila*, fez-se uso de marcadores moleculares do tipo ISSR visando contribuir para a classificação das espécies Sul Americanas. *Cunila origanoides*, espécie Norte Americana, foi usada neste trabalho para estimar a variabilidade existente entre as espécies Norte e Sul-Americanas, a qual formou um grupo geneticamente distinto das espécies Sul-Americanas. Estes dados estão de acordo com a análise filogenética do gênero. Os resultados obtidos apontam para a separação das espécies Sul Americanas em dois grupos: arbustos

e subarbustos. O grupo referente aos arbustos é formado pelas espécies que compõe as seções *Incanae* e *Incisa,* enquanto que o grupo subarbustivo é formado pelas espécies da seção *Spicatae*. Baseado na alta similaridade apresentada entre as espécies correspondentes às seções *Incanae* e *Incisae,* a união destas em uma única seção é sugerida, corroborando os dados obtidos pela análise filogenética. Este trabalho contou com a utilização de sete "primers", os quais se mostraram eficazes para a separação das espécies. Os resultados também apontam para a baixa variabilidade dentro das espécies, o que as confirma como "pools" gênicos distintos.

A seção *Incanae*, a qual se mostrou geneticamente muito similar à seção *Incisae*, conta com apenas um indivíduo, *Cunila incana*. A análise do óleo essencial desta espécie salienta um aspecto muito interessante. A percentagem de sesquiterpenos encontrada neste óleo essencial foi superior à quantidade de monoterpenos. Esta não é uma característica do gênero, sendo que todas as pesquisas realizadas com óleos essenciais de outras espécies indicaram altas concentrações de monoterpenos, e por conseqüência, poucos compostos sesquiterpênicos. Este fato sugere que a via do ácido mevalônico seja a principal rota biosintética adotada por esta espécie.

Os marcadores moleculares ISSR foram empregados no estudo de variabilidade genética dentro e entre populações de *Cunila menthoides*. As populações avaliadas formaram grupos correspondendo a sua distribuição geográfica. Cada população se caracterizou como um "pool" gênico distinto apresentando baixa variabilidade intrapopulacional, indicando que cada população é derivada de um limitado número de indivíduos com baixa troca gênica entre as diferentes populações.

Diferentes populações de *C. menthoides* foram sujeitas à hidrodestilação por arraste a vapor. Um total de 15 compostos voláteis foram detectados e identificados. Dois diferentes quimiotipos foram observados: pulegona e linalol, sendo que alguns indivíduos da população proveniente de Caçapava do Sul apresentaram variações relativas entre as percentagens de pulegona e mentona. Na via biosintética dos monoterpenos, pulegona é o composto precursor de mentona. Dependendo das condições genéticas e/ou ambientais, o composto pulegona pode ser transformado em mentona, pela

ação da enzima pulegona redutase (PR). A alta ou baixa expressão da PR pode resultar no respectivo aumento ou diminuição de produção de mentona ou pulegona, a redução do subseqüente produto acompanha a acumulação do intermediário. Possivelmente essa variação encontrada esteja ligada a variações na expressão da PR em alguns indivíduos, não caracterizando um quimiotipo. O quimiotipo linalol foi formado unicamente pela população de Barros Cassal, enquanto que todas as outras populações estudadas integraram o grupo caracterizado por pulegona.

Os estudos realizados aqui ampliam o conhecimento químico e genético deste gênero com enorme potencial aromático e medicinal. Os dados obtidos neste trabalho sugerem a afinidade entre algumas espécies, e contribuem para a elucidação da história evolutiva deste gênero, o qual possui ampla distribuição no Rio Grande do Sul.

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