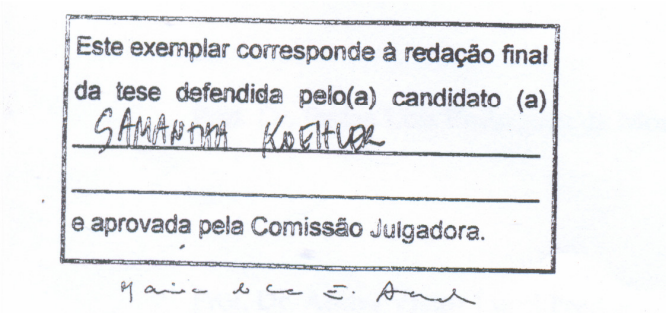
UNIVERSIDADE ESTADUAL DE CAMPINAS

SAMANTHA KOEHLER

RELAÇÕES FILOGENÉTICAS E DIVERSIFICAÇÃO NO COMPLEXO *'MAXILLARIA MADIDA'* (MAXILLARIINAE: ORCHIDACEAE)

Orientadora: Profa. Dra. Maria do Carmo Estanislau do Amaral



Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Ciências Biológicas na área de Biologia Vegetal

Campinas
2007

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

K818r

Koehler, Samantha
Relações filogenéticas e diversificação no complexo '*Maxillaria madida*' (Maxillariinae, Orchidaceae) / Samantha Koehler. – Campinas, SP: [s.n.], 2007.

Orientadora: Maria do Carmo Estanislau do Amaral.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Plantas – Análise cladística. 2. Espécies de plantas. 3. Plantas - Filogenia. 4. Marcadores genéticos. 5. Botânica - Classificação. I. Amaral, Maria do Carmo Estanislau do. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(rcdt/ib)

Título em inglês: Phylogenetic relationships and diversification within the '*Maxillaria madida*' complex (Maxilliariinae, Orchidaceae).
Palavras-chave em inglês: Plants – Cladistic analysis; Plant species; Plants – Phylogeny; Genetic markers; Plants - Classification.
Área de concentração: Biologia Vegetal.
Titulação: Doutora em Biologia Vegetal.
Banca examinadora: Maria do Carmo Estanislau do Amaral, André Olmos Simões, Cássio van den Berg, Fábio de Barros, Pedro Luís Rodrigues de Moraes.
Data da defesa: 20/04/2007.
Programa de Pós-Graduação: Biologia Vegetal.

Campinas, 20 de abril de 2007

Banca Examinadora

Profa. Dra. Maria do Carmo Estanislau do Amaral
(Orientadora)

Assinatura

Prof. Dr. André Olmos Simões

Assinatura

Prof. Dr. Cássio van den Berg

Assinatura

Prof. Dr. Fábio de Barros

Assinatura

Prof. Dr. Pedro Luís Rodrigues de Moraes

Assinatura

Prof. Dr. André Victor Lucci Freitas

Assinatura

Profa. Dra. Angela Borges Martins

Assinatura

Prof. Dr. João Semir

Assinatura

"Knowledge about the origin of life on Earth - life which, from pond serum to tigress, is connected to us through time and space - serves to inspire. That excess is natural but dangerous we learn from the photosynthetic ancestors of plants. That movement and sensation are thrilling we experience as animals. That water means life and its lack spells tragedy we garner from fungi. That genes are pooled we learn from bacteria. Modern versions of our ancient aquatic ancestors display versions of the urge to couple and of our capacity to make choices... We can ask with curiosity but answer only tentatively and with humility about the life's astonishingly rich diversity and history of change, hoping that the search continues indefinitely."

Adaptado de Margulis & Sagan (2000),
What is Life?, e Zimmer (2001),
Evolution - the triumph of an idea.

ADVERTÊNCIA

Esta dissertação não constitui publicação efetiva no sentido do artigo 29 do ICBN e, portanto, quaisquer atos nomenclaturais nela contidos tornam-se sem efeito para os princípios de prioridade e homonímia.

WARNING

This dissertation should not be considered as an effective publication in the sense of the article 29 of the ICBN, therefore, any nomenclatural acts herein proposed are considered void for the principles of priority and homonymy.

Agradecimentos

Foram muitas as pessoas e instituições que possibilitaram o desenvolvimento deste trabalho. Primeiramente, agradeço ao apoio da minha orientadora, Maria do Carmo, com quem muito aprendi nestes oito anos de convivência. Este projeto nasceu de muitas conversas com o amigo e mestre Rodrigo, que incansavelmente me ajudou a encontrar um tema para estudo e que, ao longo deste trabalho, me ensinou muito sobre orquídeas e ciência. Meu treinamento nas técnicas de obtenção e análise de seqüências de DNA e marcadores AFLP só foi possível devido a dedicação de Norris Williams, Mark Whitten, Mike Fay, Robyn Cowan e Rhian Smith. Juliano Cabral me ensinou muito sobre citogenética de orquídeas e agüentou, pacientemente, todas as minhas inúmeras perguntas básicas sobre o tema.

Muitas outras pessoas possibilitaram a realização de diferentes etapas deste trabalho, seja ajudando no trabalho de campo e/ou laboratório, viabilizando o acesso a instituições e/ou coleções de plantas, fornecendo informações valiosas que me pouparam muito tempo, ou simplesmente pelas longas e valiosas conversas sobre diferentes aspectos teóricos e práticos deste trabalho: Adal dos Santos, Ana Paula de Moraes, Anete Pereira de Souza, Anita Marsaioli, Christian Lexer, Christiano Verola, Clare Drinkell, Clarisse Palma, David Roberts, Eduardo Catharino, Elizabeth Ann–Veasey, Emerson Pansarin, Eric Christenson, Eric Smidt, Fabinho Pinheiro, Fábio de Barros, Giancarlo Oliveira, Gustavo Romero, Helena Michelan, Jeff Joseph, Joãozito Aranha, Josué Pontes, Kayna Agostini, Kurt Nubig, Lidy Aona, Lola Lledó, Ludovic Kollmann, Marcelo Guerra, Mario Blanco, Mirele Lápis, Paulo Miúdo Guimarães, Renatinho Gonçalves, Rhian Smith, Robyn Cowan, Rosana Farias–Singer, Rosana Peres, Rubens Custódio, Thelma Barbara, Seu Vitor, Volker Bittrich e Wellington Forster. Um obrigada especial também a Angelita Midori e Ricardo Lourenço por toda dedicação na elaboração dos desenhos apresentados nesta tese. O conteúdo dos capítulos aqui apresentados foi melhorado substancialmente pelos comentários de André Simões, Ângela Martins, João Semir, Maria do Carmo Amaral e Volker Bittrich.

Também gostaria de agradecer a todas as instituições que, direta ou indiretamente, colaboraram para o desenvolvimento deste trabalho: Centro de Estudos em Biologia Molecular e Engenharia Genética (CEBMEG, Unicamp), Departamento de Botânica (Unicamp), Florida Museum of Natural History (University of Florida), Ecuagenera e a família Portilla, herbários (e funcionários) BM, BR, F, HB, K, MO, NY, OUPR, P, R, S, RB, SP, UEC, US e W, Jodrell Laboratory (Kew Royal Botanic Gardens) e Orquidários do Departamento de Genética (Escola Superior de Agricultura 'Luiz de Queiroz', Esalq-USP) e do Instituto de Botânica de São Paulo.

Os estudos desenvolvidos nesta tese foram financiados pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) através da minha bolsa de doutorado (02/02161-7) e de um auxílio à pesquisa concedido a minha orientadora (02/02162-3). O primeiro capítulo desta tese também recebeu apoio financeiro da National Science Foundation (NSF, Estados Unidos) como parte do projeto '*Systematics of Maxillariinae (Orchidaceae) : generic delimitation, pollinator rewards, and pollination*' (DEB–234064). O segundo e terceiro capítulos contaram com o apoio da Kew Latin American Research Fellowship (KLARF) que me concedeu uma bolsa de estudos (Prance Fellowship in Neotropical Botany) por 12 meses para o desenvolvimento do projeto '*Species limits and phylogeography in the* 'Maxillaria acicularis – M. madida' *group*', desenvolvido em colaboração com Mike Fay (Jodrell Laboratory, Royal Botanic Gardens, Kew, Reino Unido).

Por fim, gostaria de agradecer meu marido, Glauco, e os amigos Adal, Clarisse, Fátima, Laureta, Mario Blanco, Mark, Norris, Miúdo, Rodrigo e Thelma, com quem convivi muito tempo durante o desenvolvimento desta tese, por todo carinho, apoio e dedicação às coisas boas da vida. Vocês não só tornam tudo mais divertido, como também me inspiram a querer melhorar sempre. Eu dedico este trabalho a todos vocês.

Índice

INTRODUÇÃO GERAL

Grupo de estudo

A família Orchidaceae é a maior família entre as monocotiledôneas, com cerca de 700 gêneros e 19.500 espécies, na maioria epífitas ocorrentes nos trópicos (Dressler 1993). As orquídeas apresentam tipicamente um caule tipo rizoma, com freqüência desenvolvido em pseudobulbos. As flores são geralmente zigomorfas e hermafroditas, sendo o perianto (perigônio) diferenciado em três sépalas petalóides, unidas ou livres entre si, e em três pétalas, sendo duas geralmente semelhantes entre si e às sépalas. A pétala mediana, referida como labelo, é geralmente bastante diferenciada, podendo apresentar protuberâncias e projeções de diversos tipos, usualmente denominadas calos, além de estruturas secretoras. A parte central da flor é caracterizada pela redução do número de partes florais e pela fusão dos órgãos femininos (com exceção do ovário) e masculinos em uma estrutura denominada coluna. Na maioria das espécies, o androceu é composto por um estame fértil no verticilo externo, sendo os outros estéreis ou ausentes. Neste grupo de orquídeas, os três estames do verticilo interno são modificados em estruturas (estaminóides) que variam desde pequenas protuberãncias a apêndices conspícuos da coluna. A grande maioria das espécies de orquídeas apresenta polínias, massas compactas de grãos de pólen que são removidas por polinizadores, em geral, através de estruturas acessórias que têm origem no estigma (Dressler 1993).

Os primeiros estudos em sistemática molecular de Orchidaceae basearam-se na variação entre sequências de cloroplasto com intuito de compreender a posição filogenética da família em relação à outras famílias próximas bem como entre suas subfamílias (Albert 1994, Yukawa et al. 1996, Neyland & Urbatsch 1996, Kores et al. 1997, Cameron et al. 1999). Em um estudo baseado em sequências de *rbcL* (DNA de plastídeos), Cameron et al. (1999) verificaram a monofilia das cinco subfamílias atualmente reconhecidas: (Apostasioideae (Vanillioideae [incl. Diurideae e Spiranthoideae] (Cyripedioideae (Orchidoideae (Epidendroideae [incl. orquídeas Vandoideae e Neottieae))))). Adicionalmente, regiões de rápida evolução, tais como o espaçador ITS do DNA nuclear e as regões *trnL-F* e *matK* do DNA de plastídeos, têm sido intensamente utilizadas em estudos em nível de gêneros e espécies (e.g. Bateman et al. 2003, Clements et al. 2002, Cox et al. 1997, Douzery 1999, Pridgeon et al. 1997, van den Berg et al. 2000).

Apesar do enorme avanço na classificação ao nível de subfamílias e gêneros/espécies, a compreensão das relações entre subtribos e tribos ainda demanda estudos. Por este motivo, grupos interdisciplinares constituídos por pesquisadores de diversos países têm-se unido para tentar reconstruir a filogenia das grandes tribos e subtribos de Orchidaceae (www.flmnh.ufl.edu/orchidatol/pd/orchidATOLpd.htm). Os resultados apresentados nesta tese são, em parte, fruto de uma colaboração com pesquisadores de outros países para compreensão das relações genéricas da subtribo Maxillariinae. Mais informações sobre o projeto "*Systematics of Maxillariinae (Orchidaceae): Generic delimitation, pollinator rewards, and pollination.*" podem ser obtidas no endereço: www.flmnh.ufl.edu/ herbarium/max/.

Subtribo Maxillariinae

A subtribo Maxillariinae, pertencente à tribo Cymbideae, engloba a maioria das orquídeas neotropicais e compreende de 470 espécies (Dressler 1993, Chase 2005). Esta subtribo apresenta grande diversidade vegetativa e está atualmente dividida em oito gêneros (Dressler 1993): *Anthosiphon* Schltr. (1 sp.), *Chrysocycnis* Linden & Rchb.f. (3 spp.), *Cryptocentrum* Benth. (19 spp.), *Cyrtidiorchis* Rausch. (4 spp.), *Maxillaria* Ruiz & Pavón (420 spp.), *Mormolyca* Fenzl (7 spp.), *Pityphyllum* Schltr. (4 spp.) e *Trigonidium* Lindl. (14 spp.). Em um estudo filogenético da tribo Maxillarieae, Whitten et al. (2000) demonstraram que os gêneros citados acima constituem um grupo monofilético sustentado por alto suporte estatístico de *bootstrap*. Entretanto, os resultados desse estudo também indicam que, nesse clado, a delimitação genérica atualmente adotada é insatisfatória, uma vez que grupos de espécies pertencentes ao gênero *Maxillaria* aparecem como filogeneticamente mais próximos de outros gêneros, como *Cryptocentrum* e *Trigonidium*, do que de outras espécies de *Maxillaria* (Whitten et al. 2000). *Maxillaria* (subtribo Maxillariinae) constitui um dos mais diversos gêneros neotropicais. Atualmente são reconhecidas 420 espécies mas estima-se que existam pelo menos 650 (Atwood & Mora de Retana 1999), ocorrendo desde o sul dos Estados Unidos até a Argentina. Vários estudos referentes à sistemática, morfologia e ecologia de espécies centro americanas e andinas do gênero *Maxillaria* já foram publicados (e.g. Atwood 1993; 1999, Carnevali 1996, Carnevali & Ramírez 1989), entretanto, o mesmo não se pode dizer para os representantes brasileiros do grupo (Onishi 1974, Illg 1977). Os tratamentos taxonômicos disponíveis para o gênero no Brasil (Cogniaux 1904, Hoehne

1953) são pouco úteis para identificação de espécies, principalmente por serem baseados em caracteres que variam continuamente entre os táxons reconhecidos. Pabst & Dungs (1977) propuseram uma classificação não-filogenética para orquídeas brasileiras, baseada principalmente em caracteres vegetativos, onde agruparam as espécies brasileiras de *Maxillaria* em 21 alianças. Mais de 60% das espécies de *Maxillaria* citadas por Pabst & Dungs (1977) foram indicadas como ocorrendo exclusivamente no território brasileiro, entretanto, desde o trabalho de Hoehne (1953), a grande maioria das espécies permanece pouco estudada.

O complexo 'Maxillaria madida'

Uma análise filogenética preliminar, realizada com seqüências das regiões ITS1-2 do DNA nuclear ribossomal de mais de 600 táxons da subtribo Maxillariinae, sugere que quatro alianças propostas por Pabst & Dungs (1977) para o gênero *Maxillaria* façam parte de um clado (URL: http://www.flmnh.ufl.edu/herbarium/max/, em detalhe na Fig. 1). Estas alianças foram caracterizadas por Pabst & Dungs (1977) pelo número e forma das folhas, conforme descrito abaixo:

- *Aliança 'Maxillaria madida'* (5 spp., plantas com duas folhas planas)
- *Aliança 'Maxillaria pumila'* (9 spp., plantas com uma folha plana)
- *Aliança 'Maxillaria paulistana'* (1 sp., plantas com uma folha acicular)
- *Aliança 'Maxillaria subulata'* (7 spp., plantas com duas folhas aciculares)

É importante ressaltar que as espécies pertencentes às quatro alianças de Pabst & Dungs (1977) descritas acima não constituem um grupo monofilético, uma vez que algumas espécies de *Maxillaria* ocorrentes no norte da América do Sul e na América Central também estão incluídas nesse clado (M. Whitten & N. Williams, dados não publicados). Uma das espécies amazônicas que fazem parte deste clado é *M. uncata* Lindl., também ocorrente no Brasil (região amazônica) e considerada por Pabst & Dungs (1977) como pertencente a outra aliança (aliança *'M. uncata'*), que considera apenas espécies amazônicas, não relacionadas às descritas anteriormente. *M. uncata* apresenta uma distribuição muito ampla, sendo encontrada desde a Bolívia até o sul do México. As outras espécies amazônicas pertencentes ao complexo *'Maxillaria madida'* não ocorrem no Brasil,

sendo restritas ao Equador, Peru e Bolívia. Estas são *M. nardoides* Kraenzl., *M. pacholskii* Christenson e uma nova espécie encontrada no Equador, ainda não descrita. Embora as espécies amazônicas tenham sido incluídas nos estudos taxonômico e filogenético aqui apresentados, ênfase foi dada às espécies do Sul e Sudeste do Brasil, devido à localização geográfica, disponibilidade de material para estudo e ao fato da maioria dos problemas de delimitação de espécies estarem concentrados neste grupo. Devido à ampla distribuição e plasticidade fenotípica observada em populações de *M. uncata,* Atwood & Mora de Retana (1999) sugerem que limites específicos deste táxon devem ser revistos, mas este problema não foi abordado no presente trabalho.

Apesar do complexo *'Maxillaria madida'* incluir grupos de espécies geograficamente disjuntos, essas plantas apresentam caracteres morfológicos em comum. São orquídeas de pequeno e médio porte, geralmente epífitas, raro rupícolas, que ocorrem no domínio da Mata Atlântica em localidades da Serra do Mar e da Serra da Mantiqueira ou nas florestas úmidas da região amazônica (Hoehne 1953, Onishi 1974, Pabst & Dungs 1977, Atwood & Mora de Retana 1999). Este grupo caracteriza-se por plantas que crescem geralmente em touceiras, de forma pêndula, que apresentam rizomas curtos revestidos por bainhas pardacentas, crassas e imbricantes e raízes abundantes, geralmente, anelado-rugosas.

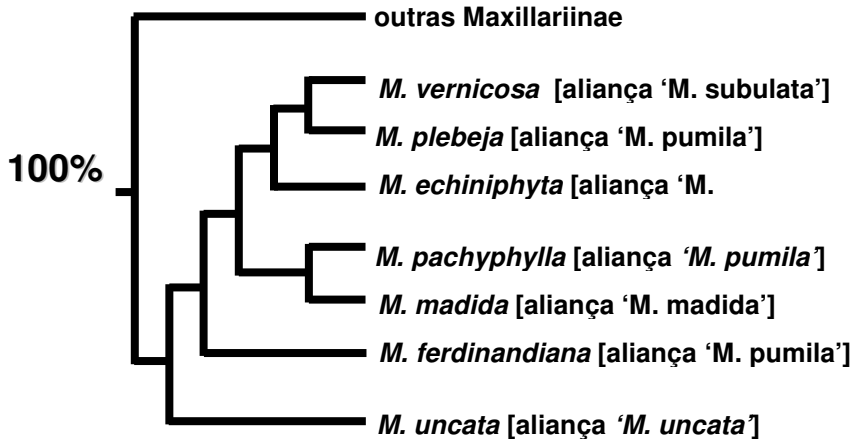


Fig. 1. Uma das árvores (parcial) de parcimônia máxima resultante do seqüenciamento das regiões ITS 1-2 do DNA ribossomal nuclear de 623 táxons representantes da subtribo Maxillariinae e grupos externos (M. Whitten et al., dados não publicados). Apenas o clado referente ao complexo *'Maxillaria madida'* está reproduzido, com valor de *bootstrap* indicado.

Os pseudobulbos geralmente são agregados, oblongos, quase roliços, apresentando 1-2(4) folhas sésseis, apicais, coriáceas a subcoriáceas, planas a aciculares. As inflorescências são extremamente reduzidas, apresentando sempre uma flor desenvolvida, geralmente de perianto carnoso e brilhante, principalmente o labelo, e de coloração alvo-amarelada, avermelhada ou castanho-escura, glabra e sem recompensa (Hoehne 1953, Onishi 1974, Davies & Turner 2004).

Histórico taxonômico

Embora o complexo *'Maxillaria madida'* seja facilmente caracterizável morfologicamente, a definição de espécies dentro do grupo tem-se mostrado bastante problemática, principalmente em relação às espécies do sudeste do Brasil. O primeiro tratamento taxonômico para o gênero *Maxillaria* no Brasil foi elaborado por Hoehne (1953). Os caracteres diagnósticos utilizados por ele para identificação de espécies incluem número, consistência e forma das folhas, assim como forma e tamanho de estruturas florais (sépalas, pétalas e labelo). Apesar da grande contribuição de sua obra, muitas das espécies aceitas por Hoehne (1953) não apresentam uma clara delimitação, em parte devido ao número restrito de espécimes disponíveis para estudo e pelo acesso restrito a materiais tipo. As formas intermediárias observadas entre espécies simpátricas do complexo *'Maxillaria madida'* foram interpretadas como prováveis híbridos pelo autor, embora tal fato nunca tenha sido demonstrado.

O estudo de Onishi (1974) representa a primeira tentativa de delimitação de um subgrupo de espécies do complexo *'Maxillaria madida'* através da realização de análises morfométricas de caracteres foliares e florais, combinadas com informações sobre a distribuição geográfica de populações. Foram analisadas 5 das 15 espécies incluídas por Butzin & Senghas (1996) no subgênero *Subulatae*, que engloba parte das espécies brasileiras do complexo *'Maxillaria madida'*. As espécies analisadas foram *M. madida* Lindl., *M. cepula* Rchb.f., *M. mosenii* Kraenzl., *M. echinochila* Kraenzl. e *M. hatschbachii* Schltr. Como não foram encontradas descontinuidades na variação morfométrica dos órgãos florais analisados, a autora concluiu que todas as espécies previamente consideradas no subgênero *Subulatae* representam um complexo polimórfico que deve ser tratado como

uma única espécie, *M. madida,* pois, na opinião bastante discutível de Onishi (1974), apenas a descontinuidade de caracteres florais poderia ser utilizada para definir espécies.

A autora propôs ainda o reconhecimento de novas subespécies e variedades, de acordo com padrões de distribuição geográfica e médias de medidas de comprimento, largura e espessura de folhas. Entretanto, além dos táxons propostos nunca terem sido efetivamente publicados, a autora propôs muitas alterações nomenclaturais sem ter examinado materiais tipo, e, portanto, as alterações propostas por ela podem não estar relacionadas aos nomes originais dos táxons. A classificação de Onishi (1974) reconheceu as seguintes subespécies e variedades para a espécie *Maxillaria madida*:

- ***M. madida* subsp. *madida***
  distribuição: norte de Santa Catarina, Paraná, São Paulo e Rio de Janeiro
  variedades: *madida*, *mosenii*, *echinochila* e *hatschbachii*;
- ***M. madida* subsp. *acicutifolia***
  distribuição: sul do Espírito Santo e sudeste de Minas Gerais
  variedades: *acicutifolia* e *rigidifolia*;
- ***M. madida* subsp. *unifolia***
  distribuição: norte do Espírito Santo ao sul da Bahia

Embora os caracteres quantitativos utilizados por Onishi (1974) para definir limites infraespecíficos tenham sido obtidos através de rigorosas análises estatísticas e da análise de vários indivíduos, eles não são úteis para identificação dos táxons aceitos pela autora, pois as descrições foram baseadas nas médias dos caracteres quantitativos obtidos, e não na amplitude de variação encontrada. Esses valores médios entre táxons são muito próximos, tornando as descrições pouco precisas, por exemplo, para a espécie *M. madida* subsp. *madida* var. *madida* para qual são referidas "folhas lineares, largamente lanceoladas". Outros caracteres, como "número de folhas por pseudobulbo" e "presença de sulcos nas folhas" sobrepõem-se em diferentes variedades e subespécies. Além disso, médias são pouco úteis para identificação de espécimes com valores distantes da média.

Talvez pelos motivos apresentados anteriormente, Pabst & Dungs (1977) não adotaram nenhum dos táxons infraespecíficos propostos por Onishi (1974), reconhecendo apenas uma única e polimórifca espécie, *Maxillaria madida*, para este grupo. É importante salientar também que o monofiletismo do subgênero *Subulatae* não foi comprovado em estudos filogenéticos preliminares (M. Whitten & N. Williams, dados não publicados) e

que o complexo *'Maxillaria madida'* inclui outras espécies morfologicamente muito semelhantes a *M. madida* e atualmente aceitas que não foram consideradas no estudo de Onishi (1974), como *M. acicularis* Herb. ex Lindl.

Em 1977, Pabst & Dungs propuseram uma classificação não filogenética baseada somente no número e forma das folhas para as espécies do complexo *'Maxillaria madida'*, que inclui as quatro alianças citadas anteriormente: aliança *'M. madida'*, que engloba a maioria das espécies classificadas no subgênero *Subulatae* (Butzin & Senghas 1996) estudado por Onishi (1974); aliança *'M. pumila'*; aliança *'M. paulistana'* e aliança *'M. subulata'*. Assim como no estudo de Onishi (1974), as alianças propostas são definidas por caracteres imprecisos, pois estes se mostram contínuos de uma aliança à outra ou extremamente variáveis como, por exemplo, os caracteres número e formato de folhas por pseudobulbo, que é polimórfico nas espécies *M. madida* e *M. cogniauxiana* Hoehne. A espécie *M. uncata* foi incluída na mesma aliança que *M. cerifera* Barb. Rodr., *M. notylioglossa* Rchb.f. e *M. johannis* Pabst por apresentar caracteres vegetativos semelhantes, como porte, rizomas longos, recobertos por brácteas e pseudobulbos unifoliados (Pabst & Dungs 1977).

Na última edição da obra *Die Orchideen* de R. Schlechter que engloba a subtribo Maxillariinae (revisada por Butzin & Senghas 1996), as espécies do complexo *'Maxillaria madida'* aparecem divididas em dois grupos principais. O grupo das plantas com folhas aciculares está representado pelas espécies *M. nardoides, M. madida, M. cogniauxiana, M. acicularis, M. juergensii* Schltr., *M. minuta* Cogn., *M. echiniphyta* Barb. Rodr., *M. vernicosa* Barb. Rodr. e *M. vitelliniflora* Barb. Rodr. e o grupo das plantas de folhas agudas engloba as espécies *M. uncata* Lindl., *M. pachyphylla* Schltr. ex Hoehne, *M. paulistana* Hoehne, *M. madida* e *M. minuta*, sendo as duas últimas citadas também para o grupo anterior. As espécies *M. madida, M. ferdinandiana* Barb. Rodr. e *M. mosenii* são citadas também no grupo de plantas com folhas escandentes. A classificação de uma mesma espécie em mais de um grupo demonstra a dificuldade em classificar espécies pertencentes a este complexo devido, principalmente, à grande variabilidade de caracteres vegetativos.

De acordo com as informações apresentadas anteriormente, fica claro que os caracteres morfológicos diagnósticos apresentados em estudos taxonômicos prévios para o gênero *Maxillaria* mostraram-se pouco eficazes para a identificação de táxons pertencentes ao complexo *'Maxillaria madida'* (Hoehne 1953, Onishi 1974, Pabst & Dungs 1977, Butzin &

Senghas 1996). Tal fato pode ser explicado principalmente pela baixa variabilidade de caracteres florais e pela variação contínua de caracteres foliares - que variam em relação à forma, consistência e número das folhas por pseudobulbo. A complexidade morfológica deste grupo indica que apenas os estudos da variação morfológica são insuficientes para esclarecer limites interespecíficos no grupo.

Nas últimas duas décadas houve um grande desenvolvimento de técnicas de biologia molecular que foram rapidamente aplicadas a estudos de sistemática, propiciando um enorme e acelerado conhecimento em biossistemática, evolução e genética de animais e plantas (e.g. Hillis et al. 1996). As técnicas moleculares estão cada vez mais acessíveis e permitem, atualmente, a comparação de regiões de DNA de diferentes indivíduos, populações e espécies e a reconstrução de filogenias robustas em diversos níveis taxonômicos. Marcadores moleculares são extremamente vantajosos pois não apresentam plasticidade fenotípica e podem ser obtidos em número elevado a partir de uma pequena quantidade de tecido. Diferentes marcadores moleculares podem apresentar taxas de evolução distintas e, através de uma escolha cuidadosa, é possível estudar diferentes aspectos da história evolutiva em diferentes níveis taxonômicos (Hillis et al. 1996).

Vários estudos têm empregado marcadores moleculares para investigar limites de espécies morfologicamente muito semelhantes e/ou que apresentam caracteres morfológicos de variação contínua, inclusive na família Orchidaceae (e.g. Borba et al. 2001, Squirrel et al. 2002). Por esse motivo, caracteres moleculares constituem ferramentas úteis para delimitação de espécies no complexo *'Maxillaria madida'*, uma vez que os caracteres morfológicos mostraram-se pouco eficazes na identificação de táxons (Hoehne 1953, Onishi 1974, Pabst & Dungs 1977). Ainda assim, análises morfológicas detalhadas são necessárias visando reavaliar caracteres tradicionalmente utilizados para delimitar táxons, bem como para definir um conjunto de caracteres morfológicos diagnósticos apropriado, que permita a identificação de espécies e grupos de espécies.

Objetivos gerais

A primeira parte do presente estudo aborda o problema de delimitação de espécies no complexo *'Maxillaria madida'* considerando todo o espectro de variação morfológica e geográfica dos táxons atualmente reconhecidos para este grupo. Os principais objetivos desta etapa foram (1) estimar as relações filogenéticas no complexo *'Maxillaria madida'* baseando-se na variação em seqüências de DNA; (2) testar a delimitação de espécies do grupo de acordo com a classificação de Hoehne 1953 com base na filogenia molecular obtida; (3) reavaliar classificações prévias propostas em nível específico para o grupo; (4) reavaliar o potencial de caracteres morfológicos na identificação de espécies.

O capítulo seguinte considera também o problema de delimitação de espécies, mas em um nível hierárquico inferior, tendo populações como unidades de estudo. Nesta etapa, foram estudadas duas espécies reconhecidas atualmente, *Maxillaria acicularis* e *M. madida*, que não apresentam caracteres morfológicos claros que permitam sua distinção. Mais do que testar os limites atualmente aceitos para duas espécies morfologicamente muito similares, este capítulo procurou investigar a influência do ambiente e do isolamento geográfico na diversificação de espécies. Para tal foram utilizados novamente marcadores moleculares, mas desta vez do tipo AFLP (*Amplified Fragment Length Polymorphism*). Os principais objetivos desta etapa foram (1) realizar um estudo comparativo baseado em marcadores AFLP de diferentes populações das espécies *M. acicularis* e *M. madida*, considerando todo o espectro de variação morfológica e geográfica observado para este grupo; (2) reavaliar conceitos taxonômicos anteriormente propostos para este clado à luz de evidências moleculares e morfológicas; e (3) investigar a influência de fatores ecológicos e geográficos potencialmente envolvidos na especiação deste grupo, relacionando dados referentes à variabilidade morfológica, distribuição geográfica, biologia reprodutiva e citogenética.

Por fim, o terceiro capítulo é uma conseqüência dos dois primeiros, uma vez que apresenta a revisão taxonômica do complexo *'Maxillaria madida'* baseada nos resultados dos capítulos anteriores. São apresentadas descrições taxonômicas completas, sinonímias, mapas de distribuição, ilustrações, além de uma chave de identificação para as dez espécies reconhecidas para o grupo.

Referências Bibliográficas

Albert VA. 1994. Cladistic relationships of the slipper orchids (Cypripediodeae: Orchidaceae) from congruent morphological and molecular data. *Lindleyana*. 9:115–132.

Atwood JT. 1993 A revision of Maxillaria neglecta complex (Orchidaceae) in mesoamerica. *Lindleyana* 8: 25-31.

Atwood JT., Mora de Retana DE. 1999. Flora Costaricencis: Orchidaceae subtribes Maxillarinae and Oncidiinae. *Fieldiana, Botany n.s.* 40: 1-182.

Bateman, RM., Hollingsworth PM,Preston J, Yi-Bo L, Pridgeon AM, Chase MW. 2003. Molecular Phylogenetics and Evolution of Orchidinae and Selected Habenariinae (Orchidaceae). *Botanical Journal of the Linnean Society* 142: 1-40.

Borba EL, Felix JM, Solferini VN, Semir J. 2001. Fly-pollinated *Pleurothallis* (Orchidaceae) species have high genetic variability: Evidence from isozyme markers. *American Journal of Botany* 88: 419-428.

Butzin F., Senghas K. 1996. Subtribus: Maxillariinae, Erster Band, Teil B. In: Brieger FG, Maatsch R, Senghas K (eds.), *Rudolf Schlechter, Die Orchideen*. Blackwell Wissenschafts-Verlag, Berlin, Wien, pp. 1727-1792.

Cameron, KM, Chase MW, Whitten WM, Kores PJ, Jarrell DC, Albert VA, Yukawa T, Hills HG, Goldman DH. 1999. A phylogenetic analysis of the Orchidaceae: evidence from rbcL nucleotide sequences. *American Journal of Botany* 86: 208–224.

Carnevali G., Ramírez I. 1989. New or Noteworthy Orchids for the Venezuelan Flora. VII. Additions in Maxillaria from the Venezuelan Guayana. *Annals of the Missouri Botanical Garden* 76:374-380.

Carnevali, G. 1996. *Systematics, phylogeny and twig epiphytism in Cryptocentrum (Orchidaceae)*. Ph.D. Dissertation, University of Missouri, St. Louis, EUA.

Chase MW. 2005. Classification of Orchidaceae in the age of DNA data. Curtis's Botanical Magazine 22(1): 2-7.

Clements MA, Jones DL, Sharma IK, Nightingale ME, Garratt MJ, Fitzgerald KJ, Mackenzie AM, Molloy BPJ. 2002. Phylogenetic systematics of the Diurideae (Orchidaceae) based on the ITS and 5.8S coding region of nuclear ribosomal DNA. Lindleyana 17: 135-171.

Cogniaux A. 1904. Orchidaceae III. Maxillarinae. In: von Martius CFP, Eichler AG, Urban I (eds.), *Flora Brasiliensis*, vol. 3(6). Frid. Fleischer, Lipsiae, pp. 1-78.

Cox AV, Pridgeon AM, Albert VA, Chase MW. 1997. Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): nuclear rDNA ITS sequences. Plant Systematics and Evolution 208: 197-223

Davies KL, Turner MP. 2004. Morphology of floral papillae in *Maxillaria* Ruiz & Pav. (Orchidaceae) *Annals of Botany* 93: 75-86.

Douzery EJP, Pridgeon AM, Kores PJ, Linder HP, Kurzweil H, Chase MW. 1999. Molecular phylogenetics of Diseae (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. *American Journal of Botany* 86: 887-899.

Dressler RL. 1993. *Phylogeny and classification of the orchid family*. Dioscorides Press, Portland.

Hillis DM, Moritz C, Mable BK. 1996. Molecular Systematics, ed. 2. Sunderland, Mass.

Hoehne FC. 1953. Orchidaceas. In: Hoehne FC, *Flora Brasilica* 10 (12, 7). Secretaria da Agricultura, São Paulo, pp. 223-341.

Illg RD. 1977. Revisão taxonômica da secção *Heterotaxis* (Lindl.) Brieg. do gênero *Maxillaria* Ruíz & Pavón. *Revista Brasileira de Biologia* 37: 281-290.

Kores PJ, Cameron KM, Molvray M, Chase MW. 1997. The phylogenetic relationships of Orchidoideae and Spiranthoideae (Orchidaceae) as inferred from *rbc*L plastid sequences. *Lindleyana* 12: 1–11.

Neyland RL, Urbatsch. 1996. Phylogeny of subfamily Epidendroideae (Orchidaceae) inferred from ndhF chloroplast gene sequences. *American Journal of Botany* 83: 1195-1206

Onishi E. 1974. Sobre o polimorfismo do grupo *Maxillaria madida* (Orchidaceae, Maxillarinae). Tese de Doutorado. Escola Superior de Agronomia Luis de Queiroz, USP, São Paulo.

Pabst GFJ, Dungs F. 1977. *Orchidaceae Brasiliensis II*. Brücke–Verlag Kurt Schmersow, Hildesheim.

Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, Chase MW. 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of Orchis sensu lato. *Lindleyana* 12: 89-109

Squirrell J, Hollingsworth PM, Bateman RM, Tebbitt MC, Hollingsworth ML 2002. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). *Molecular Ecology*11: 1957-1964.

van den berg C, Higgins WE, Dressler RL, Whitten WM, Soto Arenas M, Culham A, Chase MW. 2000. A phylogenetic analysis of Laeliinae (Orchidaceae) based on sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Lindleyana* 15: 96–114.

Whitten WM, Williams NH, Chase MW. 2000. Subtribal and generic relationship of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. *American Journal of Botany* 87: 1842-1856.

Yukawa T, Ohba H, Cameron KM, Chase MW. 1996. Chloroplast DNA phylogeny of subtribe Dendrobiinae (Orchidaceae): insights from a combined analysis based on *rbcL* sequences and restriction site variation. *Journal of Plant Research* 109: 169-176.

METODOLOGIA

Uma vez que os estudos desenvolvidos durante a elaboração desta tese estão apresentados sob a forma de artigos científicos, os métodos empregados foram redigidos de forma extremamente sucinta. Por este motivo, esta seção foi desenvolvida com intuito de apresentar em maior detalhe os métodos de obtenção e análise dos diferentes conjuntos de caracteres obtidos, com especial ênfase nos caracteres moleculares.

►Caracteres moleculares (I): seqüências de DNA

Regiões estudadas

Os caracteres utilizados para reconstrução da filogenia do clado *'Maxillaria acicularis – M. uncata'* foram seqüências de DNA referentes a quatro regiões distintas do genoma nuclear e de cloroplasto: os espaçadores ITS 1-2 (genoma nuclear); a região *trn*L-F, a região que inclui o gene *mat*K e o espaçador *atp*B-*rbc*L (genoma de plastídios). A seguir, são apresentadas informações sobre as regiões seqüenciadas. Vale lembrar que, nas matrizes de seqüências de DNA utilizadas para realização das análises filogenéticas, cada posição (ou sítio) na seqüência de DNA representa um caráter, sendo os nucleotídeos (A,C,G,T) os quatro possíveis estados (neste estudo sítios ausentes não foram considerados como caracteres válidos).

Os espaçadores internos (ITS) do DNA nuclear ribossomal (nrDNA) têm sido amplamente utilizados para o estudo das relações de parentesco de plantas, geralmente no nível genérico ou infragenérico (e.g. Hsiao et al. 1994, Sang et al. 1995, Ryan et al. 2000). Esta região inclui a unidade 5.8S - altamente conservada - e dois espaçadores denominados ITS-1 e ITS-2, que são parte integrante da unidade de transcrição do nrDNA mas não são incorporados aos ribossomos (Baldwin et al. 1995). Estudos preliminares indicam a conservação intraespecífica do comprimento das seqüências de ITS-1 e ITS-2 e alta variabilidade de seus nucleotídeos, sugerindo a utilização destes fragmentos em estudos comparativos em baixos níveis taxonômicos (Baldwin et al. 1995). Além disso, a família de genes de nrDNA, na qual os espaçadores ITS-1 e ITS-2 estão incluídos, apresenta diversas cópias por todo o genoma nuclear vegetal, permitindo a fácil detecção,

amplificação, clonagem e seqüenciamento dos fragmentos desejados (Hills & Dixon 1991, Hamby & Zimmer 1992, Baldwin et al. 1995). Este grupo de genes sofre rápida evolução combinada ("concerted evolution" sensu Arnheim et al. 1983) através de *crossing-over* desigual e conversão gênica, mecanismos que promovem uniformidade das unidades repetidas. O pequeno tamanho destes espaçadores e suas posições adjacentes a porções extremamente conservadas do genoma permitem a fácil amplificação destas seqüências através do desenho de iniciadores (*primers*) universais, conforme proposto por White et al. (1990). Apesar das limitações impostas pela interferência da estrutura secundária de nrDNA na amplificação de seqüências e da possível ocorrência de múltiplas cópias (paralogia), dados obtidos a partir de nrDNA têm se mostrado informativos para estudos filogenéticos, permitindo muitas vezes a elaboração de hipóteses de relações de parentesco entre espécies (Baldwin et al. 1995).

Diferentes regiões do DNA de cloroplasto também têm sido intensamente utilizadas para investigação de relações filogenéticas em plantas, embora a taxa de mutação relativamente baixa de muitas regiões deste genoma seja uma séria limitação no estudo de relação de parentesco no nível interespecífico (Palmer et al. 1988). Entretanto, muitas regiões não-codificadoras do cloroplasto com maior taxa de mutações têm sido identificadas nas últimas duas décadas (Palmer et al. 1988), sendo empregadas com sucesso em estudos filogenéticos em baixos níveis hierárquicos de classificação.

O típico DNA genômico de cloroplasto constitui uma molécula circular, caracterizada por dois segmentos repetidos e inversos entre si, que separam duas regiões únicas, uma mais longa e outra mais curta. As vantagens de se estudar o genoma de cloroplastos incluem seu tamanho reduzido (geralmente entre 120 e 200 kb) e o fato da maioria dos seus genes serem cópias únicas, ao contrário do ITS e de muitos outros genes nucleares, que pertencem a famílias multigênicas, conforme mencionado no item anterior. Genomas de cloroplasto também são úteis para compreender processos evolutivos em nível populacional, devido a sua herança uni parental. Entretanto, também são mais suscetíveis aos efeitos de eventos de introgressão de genoma de outras espécies através de hibridização. A introgressão de DNA entre espécies, quando não detectada, pode causar estimativas errôneas de relações filogenéticas, embora possa ser muito informativa para compreensão de processos evolutivos (Soltis & Soltis 1998).

O fragmento *trnL-trnF* é constituído de aproximadamente 1000-1200 pares de bases (em orquídeas Maxillariinae), e consiste do éxon 5'*trnL*, do íntron *trn*L, do éxon 3' *trn*L, do espaçador *trnL-F* e do éxon *trn*F (GAA) (Taberlet et al. 1991; Soltis e Soltis 1998). Taberlet et al. (1991) descreveram um conjunto de iniciadores de acordo com éxons de tRNA, que são altamente conservados em diferentes grupos de angiospermas (Bakker et al. 1999). Vários autores já demonstraram a utilidade dessa região em estudos filogenéticos em baixos níveis taxonômicos (e.g. Gielly & Taberlet 1996, Bakker et al. 1999, Whitten et al. 2000, Koehler et al. 2002).

Entre as regiões codificadoras de proteínas no genoma de cloroplastos, *matK* representa uma das que evoluem mais rápido (Wolfe 1991). Essa região está localizada na região longa de cópia única e apresenta cerca de 1550 pares de base. O gene *matK* e a região não codificadora em que está inserido podem ser facilmente amplificados através de iniciadores que anelam nas regiões extremamente conservadas que flanqueiam toda essa região, que incluem éxons *trn*K e os genes *rps16* e *psbA* (Soltis et al. 1998). Neste estudo foi amplificada a região *matK*+ intron *trnK* utilizando os iniciadores de Goldman et al. (2001), Johnson & Soltis (1994) e mais dois iniciadores para sequenciamento desenhados por M. Whitten (Florida Museum of Natural History, University of Florida, E.U.A).

A região *atpB-rbcL*, também localizada no genoma de cloroplastos, constitui um espaçador de aproximadamente 900 pares de base situado entre os genes que codificam a grande subunidade da ribulose-1,5-bifosfato-carboxilase (*rbc*L) e a subunidade dos genes da ATP-sintetase (*atp*B) (Chiang et al. 1998, Soltis et al. 1998). Apesar do comprimento desta região variar devido à ocorrência de inserções e deleções, muitas regiões conservadas, que correspondem aos promotores dos dois genes adjacentes, estão presentes (Soltis et al. 1998). Estudos prévios demonstraram a utilidade dessa região para compreender relações entre gêneros e espécies (e.g. Golenberg et al. 1993, Manen et al. 1994, Natali et al. 1995). Neste estudo, assim como para a região *matK*, foram utilizados iniciadores desenhados por M. Whitten especificamente para subtribo Maxillariinae (ver seção *Obtenção de dados* abaixo).

Coleta de material

O material utilizado para extração de DNA consistiu de folhas e flores e foi obtido, principalmente, nos orquidários do Instituto de Botânica de São Paulo (São Paulo, SP) e da Escola Superior de Agricultura "Luiz de Queiroz" (Piracicaba, SP), bem como através da coleta de espécimes em campo. Sempre que possível, foi utilizado material fresco para as extrações de DNA total, mas algumas amostras desidratadas em sílica gel também foram utilizadas para obtenção de DNA.

Obtenção de dados

Extração de DNA total[1]

O DNA total foi extraído de acordo com um protocolo modificado a partir de Doyle & Doyle (1987) para escala de 1 ml (em detalhe no anexo 1). Cerca de 50 - 100 mg de material vegetal foram macerados em 1,2 ml de solução CTAB (3% p/v) e 8 µl de 2-mercaptoetanol. Após a maceração, 1 ml do volume macerado foi aquecido a 65°C por cerca de 30 minutos. Em seguida, adicionou-se 500 µl de solução álcool isoamílico e clorofórmio (1:24), sendo as amostras em seguida centrifugadas (5 min a 10.000 r.p.m.). A precipitação foi realizada de um dia para outro a -20 °C com a adição de 500 µl de isopropanol e 30 µl de acetato de sódio ao sobrenadante obtido da centrifugação (ca. 750 µl). Posteriormente, as amostras foram centrifugadas novamente (20 min a 13.000 r.p.m.), lavadas duas vezes com etanol 70% e expostas à temperatura ambiente até estarem completamente secas. O *pellet* foi ressuspendido em 50 µl de tampão Tris-EDTA (TE) e estocado a -20°C. A quantificação de DNA extraído foi realizada através de eletroforese em gel de agarose a 0,8%-1% por comparação com marcadores de massa conhecida.

Devido à presença de compostos que interferem na reação de amplificação da maioria das espécies, o DNA total de todas as amostras obtidas foi purificado com kit de purificação e filtragem QIAquick® (Qiagen Inc.) previamente à realização das reações de amplificação. Foi seguido o procedimento conforme especificado no manual do produto, com a diferença de que a etapa de lavagem com o tampão PE foi efetuada 3 vezes.

[1] Ver protocolo de extração completo no Anexo 1.

As amostras foram ressuspendidas em tampão EB (fornecido no kit) e a presença e concentração de DNA foram verificadas novamente em gel de agarose.

Reações de amplificação[2]

As reações de amplificação das regiões ITS 1-2 e *trnL-F* foram realizadas com iniciadores descritos, respectivamente, por Sun et al. (1994) e Taberlet et al. (1991). A amplificação da região *trnL-F* foi primeiramente realizada utilizando-se os iniciadores mais externos descritos por Taberlet et al. (1991) e denominados **C** e **F**. A maioria das amostras não apresentou problemas na amplificação, entretanto para algumas a reação amplificou múltiplas bandas. Devido à possibilidade de ocorrência de polimorfismos nas regiões de anelamento dos iniciadores externos e de parálogos, as amostras que falharam foram novamente amplificadas com os iniciadores internos de Taberlet et al. (1991), denominados **E** e **D**.

Na amplificação de *matK* utilizou-se os iniciadores *matK* 308F (TAT CAG AAG GTT TTG SA) e *matK* 1100F (CAT TTC TAA TAA ATA CTC TGA C), desenhados por M. Whitten para orquídeas Maxillariinae, além de *trnK2R* (Johnson & Soltis 1995) e -19F (Goldman et al. 2001). Para amplificação do espaçador *atpB-rbcL* foram utilizados os iniciadores Max F (CTA GGT TTT GTT CTT CAA GTG TAG) e Max R (GTC AAT TTG TAA TCT TTA ACA CCA GC).

As reações utilizaram tampões e *Taq* polimerase *jumpstart* (Sigma®), 2,5 mM $MgCl_2$ (concentração final), 2,5 mM de dinucleotídeos (concentração final), além de soluções de betaína 5M (somente para amplificação de ITS 1-2) para o relaxamento da estrutura secundária do DNA (Frackman et al. 1998). Os protocolos utilizados para amplificação de cada uma das quatro regiões testadas são descritos no anexo 2. Os produtos amplificados foram purificados com o kit de purificação e filtragem QIAquick® (Qiagen Inc.) ou com o produto microCLEAN (TheGelCompany Inc.), de acordo com os protocolos dos fabricantes. O sucesso das reações de amplificação foi avaliado através de eletroforese em gel de agarose a 1%.

[2] Ver protocolos de amplificação completos no Anexo 2.

Seqüenciamento

O seqüenciamento de amostras foi realizado em seqüenciador automático (ABI 377/3100/3500, Applied Biosystems Inc.). As seqüências amplificadas foram marcadas com fluorescência através da reação de seqüenciamento constituída por 25 ciclos de 96°C/10 seg; 50°C/5 seg; 60°C/4 min. Para cada amostra foram utilizados 0,5 µl do produto *Big Dye Terminator Mix*® (Applied Biosystems Inc.), 0,5 ul do iniciador (concentração: 10 pmol/µl), 2,5 µl do tampão *Better Buffer*® (TheGelCompany Inc.), 3 µl de água e 1 µl do produto da reação de amplificação. A precipitação foi feita com etanol 95% com posterior lavagem com etanol 70% e secagem a 60°C. Ambas as fitas foram seqüenciadas para assegurar precisão na determinação de bases.

Edição de seqüências

Após a obtenção de seqüências é preciso editá-las, conferindo a identidade de cada base em ambas as fitas complementares e realizando o pareamento de fitas. Esta etapa foi realizada com o auxílio dos programas *Sequence Navigator*® e *Autoassembler*® (Applied Biosystems Inc.) e *Sequencher*® (GeneCodes Corporation).

Em seguida foi realizado o alinhamento das seqüências. Conforme citado anteriormente, os nucleotídeos das seqüências obtidas constituem os caracteres da matriz de dados. Apesar da obtenção das seqüências ser relativamente fácil, identificar quais nucleotídeos são homólogos aos outros pode não ser nada trivial, uma vez que isto é feito através do alinhamento das seqüências. O alinhamento constitui uma das mais importantes etapas da análise de seqüências para construção de filogenias, mas infelizmente esta etapa com freqüência não recebe a atenção e cuidado merecidos. Se o alinhamento for mal feito é muito provável que a se obtenha uma filogenia incorreta. Por isso, embora muitas vezes o tempo despendido em laboratório para a obtenção dos dados seja longo, é importante reservar um período para a obtenção de um bom alinhamento. Existem vários programas de alinhamento disponíveis na rede, como o CLUSTALX (Thompson et al. 1997) que são muito úteis, principalmente no alinhamento de regiões codificadoras, como *rbcL* e *matK*. Entretanto, para o alinhamento de seqüências com maior variação, é sempre bom conferir o alinhamento manualmente. Neste estudo o alinhamento foi realizado manualmente com o auxílio do programa Se-Al (Rambaut 1996).

Análises filogenéticas

Em uma análise filogenética, comparam-se os nucleotídeos localizados em um mesmo sítio das diferentes seqüências obtidas, que representam os terminais (táxons) da sua análise filogenética. Há duas explicações possíveis quando diferentes terminais apresentam determinado nucleotídeo em um mesmo sítio: os nucleotídeos são homólogos e, portanto, compartilham um ancestral comum; ou representam homoplasias, isto é, resultantes de evolução convergente, paralelismos ou reversões. A homoplasia de nucleotídeos pode ser um problema na inferência de relações filogenéticas se algumas das seqüências em estudo apresentarem taxas de substituição desiguais, ou se o método de inferência escolhido não considerar a possibilidade de substituições múltiplas. Um dos problemas mais comuns relacionados a este fenômeno é a atração de ramos longos (*long branch attraction*, Bergsten 2005), no qual, seqüências com taxas de substituição muito elevadas em relação às demais são consideradas erroneamente como filogeneticamente próximas, quando, na realidade, a similaridade da composição das suas seqüências corresponde à homoplasia. Esse problema é especialmente comum no critério de otimização de parcimônia máxima (Bergsten 2005).

Heterogeneidade de seqüências. A probabilidade de ocorrência de eventos de homoplasia é influenciada pelo padrão de substituição. Se as freqüências de nucleotídeos entre os organismos são heterogêneas, provavelmente algumas seqüências apresentam taxas de substituição maiores que as outras e, como mencionado anteriormente, isto pode levar à inferência equivocada a cerca das relações filogenéticas dos organismos em estudo. Uma forma simples de avaliar se a freqüência dos nucleotídeos em um conjunto de seqüências é heterogênea, é aplicar o teste de χ^2 de homogeneidade (Zar 1999).

Sinal filogenético. Todo e qualquer conjunto de dados, seja ele obtido ao acaso ou representante da história filogenética em um grupo de organismos, pode ser utilizado para obtenção de uma árvore filogenética. Quando a matriz a ser analisada apresenta alto grau de homoplasia é possível que as árvores resultantes não indiquem relações filogenéticas verdadeiras, mas sim que sejam produtos do ruído causado pela grande quantidade de

homoplasias. Este problema é particularmente grave quando os táxons analisados apresentam taxas de substituição muito diferentes entre si e quando o critério de otimização utilizado na análise é o de parcimônia máxima, que não considera a ocorrência de substituições múltiplas. Por esse motivo, é importante testar se os dados obtidos produzem árvores com sinal filogenético (estruturadas) ou se elas poderiam ser obtidas ao acaso. Isto pode ser feito de uma maneira muito simples. Hillis & Huelsenbeck (1992) demonstraram que a forma da curva de distribuição dos comprimentos de árvores de todas as topologias possíveis pode indicar a existência de sinal filogenético nos dados. Todo o conjunto de dados que contêm sinal filogenético produz árvores cuja distribuição de todos os comprimentos possíveis é enviesada à esquerda. A assimetria da curva de uma distribuição pode ser calculada através da estatística g_1 (Sokal & Rohlf 1981), sendo $g_1 < 0$ indicativo de curvas enviesadas à esquerda. Uma vez que, para a maioria dos conjuntos de dados, é impossível conhecer todo o conjunto de árvores possíveis, a estatística g_1 é calculada para uma amostra aleatória de árvores (geralmente entre 10^4 e 10^5), sendo posteriormente comparada com valores críticos de g_1 calculados por Hillis & Huelsenbeck (1992) para intervalos de confiança de 95% e 99%, considerando 5-25 táxons e 10-500 caracteres.

Uma vez completada a etapa de levantamento da variação de caracteres do seu grupo de interesse, como fazer para obter a filogenia? Sabemos que não é possível ter acesso a todos os organismos ancestrais e à variação de suas características para entender como a evolução ocorreu. Dispomos apenas dos dados obtidos no tempo atual para reconstruir relações de parentesco e estudar evolução de caracteres. Bem, não podemos simplesmente postular um cenário evolutivo qualquer em que determinada filogenia explique a variação encontrada no nosso conjunto de dados. Por isso, utilizamos métodos de inferência filogenética para obter árvores que representem a melhor estimativa da história evolutiva do grupo de interesse.

É importante ressaltar que todos os métodos de inferência filogenética apresentam vantagens e desvantagens. Todos eles fazem suposições quanto ao processo de evolução, mesmo que não considerem um modelo de evolução explícito, como é o caso do critério de parcimônia máxima. Também é importante lembrar que:

- É impossível saber qual é a filogenia verdadeira, por isso procuramos pela melhor estimativa de uma árvore filogenética
- A quantidade de dados disponíveis será sempre limitada
- Simulações permitem acessar os pontos vulneráveis de cada método e soluções podem ser criadas para tentar corrigi-los

Os métodos de inferência constroem árvores através de algoritmos. Algoritmos podem ser definidos com uma série específica de regras ou comandos que visam solucionar um determinado problema. Assim, embora no caso de árvores filogenéticas o problema seja o mesmo - *qual é a árvore que melhor explica a variação observada neste conjunto de dados?* - diferentes métodos utilizam diferentes algoritmos, isto é, apresentam diferentes maneiras de resolver o problema. Além disso, alguns métodos, como os que foram utilizados neste estudo, apresentam mais de uma árvore possível como solução do problema. Neste caso são utilizados critérios de otimização, que classificam, dentre todas as árvores obtidas, aquelas que representam as melhores estimativas. Os diferentes critérios priorizam diferentes aspectos de uma árvore filogenética para determinar quais são as melhores estimativas dentre todas as possíveis.

Os métodos com múltiplas soluções podem ser divididos em dois grupos principais: métodos de parcimônia e métodos baseados em "modelos de evolução". No estudo filogenético do complexo *'Maxillaria madida'* foram utilizados os critérios de otimização parcimônia máxima (MP) e o de máxima verossimilhança (ML), que são brevemente descritos a seguir.

Critério de parcimônia máxima. O princípio do critério de parcimônia máxima (MP) é identificar as topologias que exijam o menor número de eventos evolutivos para explicar as diferenças observadas entre os caracteres (e.g. seqüências de DNA) em estudo (Farris 1970). Assim como os outros critérios, a parcimônia apresenta vantagens e desvantagens que devem ser consideradas em todo estudo filogenético. Dentre as vantagens do critério MP pode-se citar a facilidade de compreensão e o fato de considerar relações ancestral-descendente, ao contrário dos métodos fenéticos (e.g. UPGMA) que agrupam terminais

apenas de acordo com a similaridade. Além disso, o critério de MP avalia soluções múltiplas e pode ser utilizado para inferir informação em táxons ancestrais. Entretanto, em geral, as estimativas de comprimento de ramos fornecidas pelo critério de parcimônia tendem a ser menores que os valores reais, principalmente quando a divergência entre as seqüências é elevada. A confiabilidade do critério MP também diminui quando há ocorrência de substituições reversas, substituições paralelas (homoplasias), poucos sítios informativos, variância da taxa de ocorrência de eventos de substituição ao longo de uma linhagem e divergência acentuada entre as seqüências de atributos nos terminais (Nei 1991). Outra dificuldade é que os resultados extraídos da aplicação do critério MP não são diretamente tratáveis por ferramentas estatísticas básicas, pois não há métodos simples para se obter, por exemplo, média e variância da parcimônia de uma árvore. Quando mais de uma proposta de topologia apresenta a mesma parcimônia, não é possível decidir por uma delas com base no critério de parcimônia. Sendo assim, todas as topologias igualmente parcimoniosas são consideradas potencialmente corretas. Neste caso, geralmente é apresentada uma árvore de consenso (Swofford & Begle 1993). Computacionalmente, análises que consideram o critério MP tendem a ser mais rápidas do que aquelas que utilizam o critério de verossimilhança máxima, mas mais lentas do que métodos de distância, que constituem métodos de solução única.

Todas as análises filogenéticas que consideraram o critério de parcimônia neste estudo foram realizadas com o programa PAUP 4.0b10 (Swofford 2000). Foram considerados caracteres não ordenados e peso iguais para todas as mudanças (Fitch 1971). As análises consideraram apenas caracteres informativos e otimização do tipo ACCTRAN (ACCelerated TRANsformation). Foram obtidas 10.000 árvores iniciais através do algoritmo *stepwise addition* com adição aleatória de táxons, salvando 10 árvores ótimas/réplica. O algoritmo de *branch swapping* utilizado foi o *tree-bisection-reconnection* (TBR), salvando-se todas as árvores mínimas possíveis (opção MULTREES). De forma a reduzir o tempo de busca, foram salvas apenas 10 árvores/réplica. A consistência dos ramos foi testada com o procedimento de reamostragem de *bootstrap* (Felsenstein 1985), utilizando 1000 pseudo-réplicas, com 10 réplicas de adição aleatória de taxa e salvando 1 árvore/réplica. As categorias arbitrárias utilizadas para descrever o suporte dos ramos

foram: sem suporte (<50%); baixo suporte (50-74%); suporte moderado (75-84%); alto suporte (85-100%).

Critério de verossimilhança máxima. A verossimilhança máxima (ML, Fisher 1922) é um critério alternativo ao de parcimônia para escolher a árvore filogenética ótima dentre todas as possíveis. Verossimilhança nada mais é do que a probabilidade de ocorrência de um determinado conjunto de dados que variam de acordo com um modelo probabilístico. Em sistemática molecular, os dados referem-se às seqüências de DNA alinhadas, e o modelo a uma hipótese de evolução – que corresponde à topologia da árvore (ou, mais precisamente, aos comprimentos de ramos) e ao mecanismo da mudança evolutiva, ou o modelo de evolução propriamente dito, que inclui informações tais como a freqüência de ocorrência de cada nucleotídeo, a taxa de substituição/sítio e como as taxas de substituição variam em diferentes nucleotídeos. As análises de ML foram realizadas também com o programa PAUP.

O modelo de evolução escolhido tem uma influência enorme na probabilidade dos dados e, conseqüentemente, no resultado da análise (Foster 2001). Por isso, em toda análise filogenética que considera métodos baseados em modelos de evolução, é muito importante selecionar o modelo mais apropriado para o conjunto de seqüências obtido. Neste estudo, a etapa de seleção de modelos foi feita através do teste de razão da verossimilhança, que compara pares de modelos hierárquicos aninhados através de um teste de hipóteses (Johnson & Omland 2004). Neste estudo, a seleção de modelos foi feita de acordo com o programa Modeltest (Posada & Crandall 1998). Os parâmetros obtidos para as análises realizadas foram: modelo *General Time Reversable*, freqüências nucleotídicas estimadas A = 0,21 C = 0,28 G = 0,32 T = 0,19; valor α (distribuição γ) = 0,47 e –lnL = 2528,53 [ITS]; modelo F81+G+I, freqüências nucleotídicas estimadas A = 0,32 C = 0,15 G = 0,14 T = 0,39; α (distribuição γ) = 0,90; proporção de sítios invariantes (pinvar) = 0.61 e –lnL = 2528,53 [plastídeos].

►Caracteres moleculares (2): marcadores AFLP

A técnica de polimorfismos de comprimento em fragmentos amplificados, ou *'Amplified Fragment Length Polymorphisms'* (AFLP) foi inicialmente desenvolvida para construção de mapas genéticos (Vos et al. 1995). No entanto, devido a sua grande eficácia em identificar um grande número de polimorfismos, sua aplicação em estudos de sistemática molecular e genética de populações foi quase imediata (e.g. Janssen et al. 1996, Hill et al. 1996). Outras grandes vantagens desta técnica são: (1) não é necessário ter conhecimento prévio da identidade de seqüências das regiões que serão amplificadas; (2) a otimização da técnica e a obtenção dos marcadores é, geralmente, relativamente rápida quando comparada a outros marcadores, como microssatélites; (3) um grande número de locos distintos pode ser analisado simultaneamente, sendo o genoma como um todo densamente amostrado; (4) os resultados são facilmente reproduzidos, ao contrário de marcadores RAPDs; (5) é possível utilizar amostras desidratadas em sílica gel para extração de DNA, permitindo o estudo de organismos de difícil cultivo (Harris & Robinson 1994, Mace et al. 1999, Mueller & Wolfenbarger 1999, Russell et al. 1999).

A maior limitação dessa técnica em estudos de biologia comparada é, possivelmente, identificar a homologia entre fragmentos. Dois fragmentos de mesmo tamanho podem não ser homólogos mas sim representar seqüências distintas mas de mesmo comprimento (Hillis 1994). Da mesma forma, a evolução de inserções e deleções entre sítios de restrição pode levar à identificação errônea de dois fragmentos homólogos como heterólogos.

Entretanto, apesar dos problemas acima mencionados, numerosos estudos filogenéticos utilizando marcadores AFLP indicaram relações congruentes com dados morfológicos e/ou com seqüências de DNA (e.g. Kardolus et al. 1998, Mace et al. 1999, Goldman et al. 2004). A princípio, quando marcadores AFLP são comparados entre táxons filogeneticamente muito próximos (dentro de uma mesma espécie ou em complexos de espécies), o ruído causado pela homoplasia não é grande o suficiente a ponto de inviabilizar a aplicação deste marcador em estudos comparativos (El-Rabey et al. 2002).

Coleta de material

Assim como o material coletado para seqüenciamento, as amostras utilizadas na obtenção de marcadores AFLP consistiram de folhas e flores frescas coletadas nos orquidários do Instituto de Botânica de São Paulo e da Escola Superior de Agricultura "Luiz de Queiroz" e de plantas em cultivo originárias de coletas próprias. Algumas amostras desidratadas em sílica gel também foram utilizadas.

Obtenção de dados

Extração de DNA total

O protocolo para extração de DNA foi o mesmo utilizado no seqüenciamento de DNA, que foi descrito no item anterior. Entretanto, para que a técnica de AFLP seja otimizada, é aconselhável purificar o DNA para eliminar resíduos de etanol e outros compostos inibidores que podem interferir no desenvolvimento da técnica. Todas as amostras utilizadas para este estudo foram purificadas com kit de purificação e filtragem QIAquick® (Qiagen Inc.), seguindo o protocolo recomendado pelo fabricante.

Reação de digestão-ligação [3]

A primeira etapa da técnica para obtenção de marcadores AFLP consiste de duas reações: digestão e ligação, que são realizadas simultaneamente. Na reação de digestão, enzimas de restrição[4] são utilizadas para gerar inúmeros fragmentos a partir do DNA total extraído. A digestão na técnica de AFLP é feita geralmente com as enzimas de restrição *Mse*I (denominada enzima de corte freqüente, com 4 pares de base; pb) e *Eco*RI (enzima de corte raro, com 6 pb). Além da digestão, é necessário realizar a ligação dos fragmentos de DNA resultantes da digestão com adaptadores, que nada mais são do que seqüências constituídas por bases complementares às regiões clivadas pelas enzimas de restrição mais um pequeno fragmento de seqüências conhecidas. As reações de digestão-

[3] Ver protocolo completo no Anexo 4.

[4] Uma enzima de restrição ou endonuclease de restrição é um tipo de nuclease que cliva uma fita dupla de DNA sempre que identificar uma seqüência particular de nucleotídios, que é geralmente palindrômica e conhecida (Smith & Wilcox, 1970).

ligação foram realizadas em um termociclador a 37º C por 2h, sendo o sucesso da reação verificado em gel de agarose 1,5% (ver Anexo 4 para protocolo).

Reações de amplificação

Na etapa següinte foram realizadas as reações de amplificação pré-seletiva e seletiva. Na amplificação pré-seletiva, as seqüências conhecidas dos adaptadores funcionam como sítios de anelamento para iniciadores específicos, mas apenas aqueles fragmentos que apresentam adaptadores em ambas as extremidades são amplificados em abundância. Além disso, para reduzir o número de fragmentos que serão amplificados, os iniciadores utilizados na amplificação pré-seletiva apresentam um nucleotídeo extra. No final da reação apenas uma parte dos fragmentos obtidos na reação de digestão-ligação terá sido amplificada. O sucesso da reação de amplificação pré-seletiva foi também verificado através de um gel de agarose 1%-2% (ver Anexo 4 para protocolo).

Mesmo tendo amplificado apenas uma fração dos fragmentos resultantes da digestão, o número de fragmentos resultantes da reação de amplificação pré-seletiva ainda é muito grande para ser quantificado em um gel de poliacrilamida, sendo necessário realizar uma segunda reação de amplificação, desta vez mais seletiva do que a primeira. Nesta reação, denominada de amplificação seletiva, geralmente 2-3 combinações de iniciadores são utilizadas, sendo cada combinação formada por dois iniciadores, um baseado na seqüência da enzima *Eco*RI e outro na da enzima *Mse*I. Entretanto, para plantas com genomas tamanho padrão, existem oito iniciadores possíveis para cada enzima (sendo 64 as combinações possíveis). É necessário, portanto, realizar uma análise piloto para selecionar quais combinações de iniciadores são mais apropriadas para os organismos em estudo. A análise piloto realizada para este estudo considerou cinco amostras distintas e 12 combinações de iniciadores, sendo selecionadas três combinações (ACT-CAC; AGG-CAA; AAC-CAC) que apresentaram o grau de variação mais apropriado para este estudo, sendo que apenas duas resultaram em eletroferogramas de boa qualidade. O protocolo utilizado na reação de amplificação seletiva é apresentado no Anexo 4.

Seqüenciamento e edição de dados

Em seguida, os produtos da reação de amplificação seletiva foram processados em um seqüenciador automático (ABI 3100, Applied Biosystems Inc.), sendo os eletroferogramas visualizados e editados com auxílio dos programas Genotyper® (Applied Biosystems Inc.) e Microsoft Excel® (Microsoft Corporation). A presença/ausência de cada fragmento foi verificada comparativamente em todas as amostras, de forma a garantir precisão da matriz de dados utilizada nas análises estatísticas.

Análises estatísticas

Conforme discutido anteriormente, eventos de homoplasia e de não-independência entre fragmentos devem ser considerados na análise de dados de AFLP. Uma vez que fragmentos de AFLP foram convertidos em uma matriz, os dados podem ser analisados com duas abordagens distintas: análises multivariadas e análises filogenéticas.

(i) Análises multivariadas

Análises multivariadas referem-se a um conjunto de métodos estatísticos utilizado para estudar as relações entre múltiplas variáveis simultaneamente (Fowler et al. 1998). A essência desta metodologia é reduzir a complexidade do conjunto de dados obtido, geralmente constituído por um grande número de variáveis, de forma a facilitar a identificação de padrões de variação. Entretanto, existem diversos tipos de algoritmos distintos disponíveis para realização de análises multivariadas, cada um com pontos fortes e fracos. Por este motivo, é interessante utilizar métodos multivariados distintos e comparar os resultados obtidos. Para este estudo foram utilizadas análises de agrupamento e os métodos de ordenação, que são resumidamente descritos a seguir.

Neighbor-joining. Análise agrupamento, como o próprio diz, é o termo genérico aplicado a qualquer técnica que tem como objetivo identificar agrupamentos de unidades amostrais onde o número total de agrupamentos é desconhecido (Fowler et al. 1998). Neighbor-joining (Saitou & Nei 1987) é um método de agrupamento que infere uma árvore (no caso, dendrograma) a partir de uma matriz de distâncias genéticas através do agrupamento sucessivo de pares de terminais. Assim, a matriz original com dados de presença/ausência de fragmentos de AFLP é transformada em uma matriz de distâncias genéticas corrigidas de acordo com um modelo de evolução apropriado para o conjunto de dados em estudo. Para correção das distâncias calculadas para presença/ausência de sítios de restrição foi aplicado o coeficiente de Nei & Li (1979), que estima a proporção apenas de fragmentos compartilhados (e não os ausentes) por dois indivíduos considerando que esses indivíduos compartilham um ancestral comum mais próximo. Os agrupamentos obtidos no dendrograma resultante são então comparados com a classificação disponível. Novas classificações também podem ser propostas com base no dendrograma obtido, mas não existe uma regra para quantos agrupamentos reconhecer em um dendrograma. Geralmente, são reconhecidos os agrupamentos mais estáveis, definidos por distâncias comparativamente maiores. A consistência dos agrupamentos obtidos foi avaliada através do procedimento de reamostragem *bootstrap* (Efron & Tibshirani 1993) utilizando-se 1000 pseudo-réplicas.

Análise de Coordenadas Principais (PCoA). O objetivo principal da análise de coordenadas principais é reduzir a complexidade dos resultados obtidos através da identificação de conjuntos de fragmentos que explicam a maior parte da variação observada entre os indivíduos amostrados (Fowler et al. 1998). Cada conjunto de fragmentos, obtido através da comparação de valores de similaridade entre indivíduos e que explica uma parte da variação observada, é denominado de coordenada principal. A primeira coordenada principal é aquela que explica a maior parte dos padrões de divergência obtidos. A segunda coordenada principal deve ser o mais diferente possível da primeira, explicando a segunda maior quantidade de variação encontrada. A vantagem da utilização de coordenadas é que elas não são correlacionadas, isto é, devem ser o mais possivelmente distintas entre si. Em geral, o número total de coordenadas principais a ser

considerado restringe-se a algumas poucas (cerca de 3-4), uma vez que explicam a grande maioria da variação observada ou que contém uma quantidade de informação maior que a média obtida para todas as coordenadas (Fowler et al. 1998). Quando as primeiras coordenadas (e.g. 2-4) explicam grande parte da variação observada, é possível visualizar a relação entre duas coordenadas principais em um gráfico de dispersão. Entretanto, se as primeiras coordenadas não explicam muito a variação original observada entre os indivíduos, é provável que a análise de poucas coordenadas principais indique relações equivocadas. Uma maneira de evitar esta distorção é considerar todas as coordenadas com auto-valores maiores que 1.0 (Iezzoni & Prittis 1991). As medidas de similaridade utilizadas para análise de coordenadas principais deste estudo foram obtidas com o coeficiente de Jaccard (Jaccard 1908), que considera apenas os fragmentos presentes.

(ii) Análises de *Bootstrap*

Independente do método escolhido para a análise de dados em um estudo filogenético ou de similaridade genética é muito importante que tenhamos uma medida de confiabilidade das relações resultantes. Medidas de sustentação para os clados internos podem ser obtidas através de técnicas de reamostragem, tais como *jackknife* e *bootstrap* (Soltis & Soltis 2003). No presente estudo utilizamos a técnica de reamostragem de *bootstrap* (Efron & Tibshirani 1993, Felsenstein 1985) para avaliar a sustentação interna das filogenias obtidas.

A técnica de *bootstrap* aplicada à inferência filogenética consiste em reamostrar, aleatoriamente e com reposição, os caracteres, isto é, as colunas da matriz de dados original. A nova matriz reconstruída com os caracteres reamostrados é utilizada para realização de uma nova análise (e.g. inferência filogenética ou UPGMA). Este processo é repetido muitas vezes, geralmente 500-1000, de modo que ao término da análise teremos obtido uma nova árvore para cada reamostragem de *bootstrap* realizada. Em seguida, utilizam-se todas as árvores de *bootstrap* obtidas para construção de uma árvore de consenso, onde geralmente apenas os clados/agrupamentos que apareceram em mais de 50% das árvores obtidas (e.g. com valores de *bootstrap* maiores que 50%) são mostrados. Para cada um desses clados/agrupamentos da árvore de consenso é indicado o valor de *bootstrap* correspondente - assim, se um determinado clado/agrupamentos foi obtido em

99% de todas as 1000 árvores de *bootstrap*, ele vai apresentar um valor de 99 na árvore de consenso de *bootstrap.*

►Caracteres morfológicos

O estudo de caracteres morfológicos baseou-se, quase exclusivamente, em material fresco, obtido de espécimes em cultivo no Instituto de Botânica de São Paulo, na Escola Superior de Agricultura "Luiz de Queiroz" e na Universidade Estadual de Campinas. Amostras de raízes, pseudobulbos, folhas e flores foram coletadas para estudos morfológicos e fixados em etanol 70%. Para melhor compreensão da variabilidade de caracteres morfológicos, assim como para determinação dos padrões de distribuição geográfica das espécies deste grupo, também foi solicitado o empréstimo de exsicatas a herbários nacionais (R, RB, HB, SP, INPA, MG, MBML, CVRD) e internacionais (F, K, MO, NY, US) por apresentarem coleções importantes de orquídeas neotropicais. As medidas apresentadas neste estudo foram obtidas com auxílio de um paquímetro digital Mitutoyo 200 mm.

Referências bibliográficas

Arnheim N. 1983. Concerted evolution in multigene families. In: Nei M, Koehn R (eds.), *Evolution of Genes and Proteins*. Sinauer Associates, Sunderland, Massachusetts, pp. 38-61.

Bakker FT, Culham A, Gibby M. 1999. Phylogenetics and diversification in *Pelargonium*. In: Hollingsworth PM, Bateman RM, Gornall RJ (eds), *Molecular systematics and plant evolution*. Taylor & Francis, London, pp. 353-374.

Baldwin BG., Sanderson MJ, Porter JM, Wajciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribossomal DNA: a valuable source of evidence of angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247-277.

Bergsten J. 2005. A review of long-branch attraction. *Cladistics* 21: 163-193.

Butzin F., Senghas K. 1996. Subtribus: Maxillariinae, Erster Band, Teil B. In: Brieger FG, Maatsch R, Senghas K (eds.), *Rudolf Schlechter, Die Orchideen*. Blackwell Wissenschafts-Verlag, Berlin, Wien, pp. 1727-1792.

Chiang TY, Schaal BA, Peng C-I. 1998. Universal primers for amplification and sequencing a non-coding spacer between the atpB and rbcL genes of chloroplast DNA. *Botanical Bulletin of Academica Sinica* 39: 245-250.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin of the Botanical Society of America* 19: 11-15.

Efron B., Tibshirani R. 1993. *An introduction to the Bootstrap*. Chapman and Hall, New York.

El-Rabey HA, Badr A, Schäfer-Pregl R, Martin W, Salamini F. 2002. Speciation and species separation in *Hordeum* L. (Poaceae) resolved by discontinuous molecular markers. *Plant Biology* 4: 567–575.

Farris JS. 1970. Methods for computing Wagner trees. *Systematic Zoology* 19: 83-92.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

Fisher RA. 1922. On the mathematical foundations of theoretical statistics. *Philosophical Transactions of the Royal Society, ser. A* 222:309-368

Fitch WM. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* 20: 406-416.

Foster PG. 2001. The idiot's guide to the zen of likelihood in a nutshell in seven days for dummies, unleashed. www.ucalgary.ca/~dsikes/.

Fowler J, Cohen L, Jarvis P. 1998. *Practical statistics for field biology*. 2nd edition. John Wiley & Sons, New York.

Frackman S. 1998. Betaine and DMSO: Enhancing agents for PCR. *Promega Notes* 65: 27–30.

Gielly L, Taberlet P. 1996. A phylogeny of the European gentians inferred from chloroplast trnL (UAA) intron sequences. *Botanical Journal of the Linnean Society* 120: 57-75.

Goldman DH, Jansen RK, van den Berg C, Leitch IJ, Fay, MF, Chase MW. 2004. Molecular and cytological examination of *Calopogon* (Orchidaceae, Epidendroideae): circumscription, phylogeny, polyploidy, and possible hybrid speciation. *American Journal of Botany* 91: 707-723.

Golenberg EM, Clegg MT, Durbin ML, Doebley J, Ma DP. 1993. Evolution of a noncoding region of the chloroplast genome. *Molecular Phylogenetics and Evolution* 2, 52–64.

Hamby RK, Zimmer EA. 1992. Ribosomal RNA as a phylogenetic tool in plant systematics. In: Soltis P, Soltis D, Doyle JJ (eds.), *Molecular Systematics of Plants*. Chapman and Hall, New York and London, pp. 50-91.

Harris SA, Robinson J. 1994. Preservation of Tropical Plant Material for Molecular Analyses. In: Adams RP, Miller JS, Golenberg EM, Adams JE (eds.), *Conservation of Plant Genes II. Monographs in Systematic Botany from the Missouri Botanical Garden 48*. Missouri Botanical Garden, St. Louis, pp. 83-92.

Hill M, Witsenboer H, Zabeau M, Vos P, Kesseli R, Michelmore R. 1996. PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics* 93: 1202 – 1210.

Hillis DH, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83: 189-195.

Hillis DM. 1994. Homology in molecular biology. In: Hall BK. (ed.), *Homology, the Hierarchical Basis of Comparative Biology*. Academic Press, San Diego, pp. 339-368.

Hills DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology* 66: 411-453.

Hoehne FC. 1953. Orchidaceas. In: Hoehne FC, *Flora Brasilica* 10 (12, 7). Secretaria da Agricultura, São Paulo, pp. 223-341.

Hsiao C, Chatterton KH, Jensen KB. 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome* 37: 112-120.

Iezzoni AF, Prittis MP. 1991. Applications of principal component analysis to horticultural research. *Hortscience* 26: 334-338.

Illg RD. 1977. Revisão taxonômica da secção *Heterotaxis* (Lindl.) Brieg. do gênero *Maxillaria* Ruíz & Pavón. *Revista Brasileira de Biologia* 37: 281-290.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. *Bulletin de la Société Vaudense des Sciences Naturelles* 44: 223-270.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M, Kersters K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142: 1881-1893.

Johnson JB, Omland KS. 2004. Model selection in ecology and evolution. *Trends in Ecology and Evolution* 19: 101-108.

Johnson LA, Soltis DE. 1995. Phylogenetic inference in Saxifragaceae *sensu stricto* and *Gilia* (Polemoniaceae) using matK sequences. *Annals of the Missouri Botanical Gardens* 82: 149-175.

Kardolus JP, van Eck HJ, van den Berg RG. 1998. The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (Solanaceae). *Plant Systematics and Evolution* 210: 87-103.

Koehler S, Williams NH, Whitten WM, Amaral MCE. 2002. Phylogeny of the *Bifrenaria* (Orchidaceae) complex based on morphology and sequence data from nuclear rDNA internal transcribed spacers (ITS) and chloroplast trnL-trnF region. *International Journal of Plant Sciences* 163: 1055-1066.

Mace ES, Lester RN, Gebhardt CG. 1999. AFLP analysis of genetic relationships among the cultivated eggplant, *Solanum melongena* L. and wild relatives (Solanaceae). *Theoretical and Applied Genetics* 99: 626–633.

Manen JF, Savolainen V, Simon P. 1994. The atpB and rbcL promoters in plastid DNAs of a wide dicot range. *Journal of Molecular Evolution* 38: 577–582.

Mohammadi SA, Prasanna BM. 2003. Analysis of genetic diversity in crop plants – salient statistical tools and considerations. *Crop Sciences* 43: 1235-1248.

Mueller U., Wolfenbarger L. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* 14: 389–394.

Natali A, Manen JF, Ehrendorfer F. 1995. Phylogeny of the Rubiaceae–Rubioideae, in particular the tribe Rubieae: evidence from a non-coding chloroplast DNA sequence. *Annals of the Missouri Botanic Garden* 82: 428–439.

Nei M. 1991. Relative efficiencies of different tree-making methods for molecular data. In: Miyamoto MM, Cracraft J. (eds.), *Phylogenetic analysis of DNA sequences*. Oxford University Press, New York, pp. 90-128.

Nei M, Li W-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science* 76: 5269-5273

Onishi E. 1974. Sobre o polimorfismo do grupo *Maxillaria madida* (Orchidaceae, Maxillarinae). Tese de Doutorado. Escola Superior de Agricultura Luiz de Queiroz, USP, São Paulo.

Pabst GFJ, Dungs F. 1977. *Orchidaceae Brasiliensis II*. Brücke–Verlag Kurt Schmersow, Hildesheim.

Palmer JD, Jansen RK, Michaels HJ, Chase MW, Manhart JR. 1988. Chloroplast DNA variation and plant phylogeny. *Annals of the Missouri Botanic Garden* 75: 1180-1206.

Posada D, Crandall KA 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Rambaut, A. 1996. *Se-Al: Sequence Alignment Editor*. Available at http://evolve.zoo.ox.ac.uk/.

Russell JR, Weber JC, Booth A, Powell W, Sotelo-Montes C, Dawson IK. 1999. Genetic variation of *Calycophyllum spruceanum* in the Peruvian Amazon Basin, revealed by amplified fragment length polymorphism (AFLP) analysis. *Molecular Ecology* 8: 199-204.

Ryan A, Whitten WM, Johnson MAT, Chase MW. 2000. A phylogenetic assessment of *Lycaste* and *Anguloa* (Orchidaceae: Maxillarieae). *Lindleyana* 15: 33-45.

Saitou N. & Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Sang T, Crawford DJ, Silva M. 1995. ITS sequences and the phylogeny of the genus *Robinsonia* (Asteraceae). *Systemtic Botany* 20: 55-64.

Smith HO, Wilcox KW. 1970. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *Journal of Molecular Biology* 51(2):379–391.

Sneath PHA, Sokal RR. 1973. *Numerical taxonomy*. W. H. Freeman, San Francisco.

Sokal RR, Rohlf FJ. 1981. *Biometry, 2nd edition*. W.H. Freeeman, San Francisco.

Soltis DE, Soltis PS. 1998. Choosing an approach and an appropriate gene for phylogenetic analysis. In: Soltis DE, Soltis PS, Doyle J J (eds.), *Molecular Systematics of Plants II. DNA sequencing*. Kluwer Academic Publisher, Boston, Dordrecht, London, pp.1-43.

Soltis DE, Soltis PS, Mort ME, Chase MW, Savolainen V, Hoot SB, Morton CM. 1998. Inferring complex phylogenies using parsimony: an empirical approach using three large DNA data sets for angiosperms. *Systematic Biology* 47: 32-42.

Soltis DE, Soltis PS. 2003. The role of phylogenetics in comparative genetics. *Plant Physiology* 132: 1790–1800

Sun Y, Skinner DZ, Liang GH, Hulbert SH. 1994.Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribossomal DNA. *Theoretical and Applied Genetics* 89: 26-32.

Swofford DL 2000. *PAUP*: Phylogenetics analysis using parsimony (and other methods), version 4.0b10*. Sinauer Associates, Sunderland, Massachusetts.

Swofford DL, Begle DP. 1993. User's Manual for *PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.* Illinois Natural History Survey, Champaign, IL, USA.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds.). *Molecular Systematics, 2nd edition.* Sinauer Assoc. Inc., Massachusetts, pp. 407-514.

Taberlet PL, Geilly G, Bouvet J. 1991. Universal primers for amplification on three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105-1109.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgings DG. 1997. The clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.

Wolfe KH. 1991. Protein-coding genes in chloroplast DNA: compilation of nucleotide sequences, data base entries and rate of molecular evolution. In: Vasil K (ed.), *Cell culture and somatic cell genetics of plants, 7 BI* . Academic Press, San Diego, California, USA, pp. 467-482.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribossomal RNA genes for phylogenies. In: Innis M., Gelfand D., Sninsky J., White TJ (eds.), *PCR Protocols: A guide to methods and applications*. Academic Press, San Diego, California, pp: 315-322.

Whitten WM, Williams NH, Chase MW. 2000. Subtribal and generic relationship of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. *American Journal of Botany* 87: 1842-1856.

Zar JH. 1999. *Biostatistical analysis.* Prentice Hall, Upper Saddle River, New Jersey

.

1

FILOGENIA MOLECULAR E DELIMITAÇÃO DE ESPÉCIES DO COMPLEXO '*MAXILLARIA MADIDA*' BASEADA EM SEQÜÊNCIAS DE DNA

Molecular Phylogeny of the Neotropical *'Maxillaria madida'* Complex (Orchidaceae): Species Delimitation and insights into Chromosome Evolution

Samantha Koehler [1, 2*], Juliano S. Cabral [3], W. Mark Whitten [4], Norris H. Williams [4], Rodrigo B. Singer [5], Kurt M. Neubig [4], Marcelo Guerra [3], Anete P. Souza [6], Maria do Carmo E. Amaral [2]

[1] Pós–graduação em Biologia Vegetal and

[2] Departamento de Botânica, IB, Universidade Estadual de Campinas, Campinas, SP, 13083–970, Brazil

[3] Departamento de Botânica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife, PE, 50670–420, Brazil

[4] Florida Museum of Natural History, University of Florida, Dickinson Hall, Gainesville, FL, 32611–7800, U.S.A.

[5] Departamento de Botânica, IB, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91501–970, Brazil.

[6] Departamento de Genética e Evolução, IB, Universidade Estadual de Campinas, Campinas, SP, 13083–970, Brazil

Key words: atpB–rbcL spacer, ITS, *matK-trnK* intron, *trnL–F*, Maxillariinae, molecular phylogenetics, chromosome number, cytotaxonomy, fluorochrome staining.

Abstract

Species concepts have varied largely in the '*Maxillaria madida*' complex due to the high polymorphism of morphological characters and phenotypic plasticity. To assess species boundaries and provide a more stable classification for this group, we proposed to infer phylogenetic relationships based on sequence data of multiple regions; to provide data on chromosome counts and heterochromatin distribution patterns and to describe sets of diagnostic morphological characters for species identification. Phylogenetic studies were based on sequence data of plastid *atpB-rbcL* spacer, *trnL-F*, *matK* + *trnK* intron, and ITS of 51 samples, using the maximum parsimony and maximum likelihood criteria. Chromosome numbers and heterochromatin distribution patterns based on CMA and DAPI fluorochromes were obtained for eight taxa. We propose the recognition of nine species according to the results of the molecular phylogeny allied to the distribution of morphological characters. Six currently accepted species with good morphological diagnostic characters were recovered by both nuclear and plastid DNA sequence phylogenies. Molecular and morphological data also suggest that the '*M. pumila*' clade should be recognized as a single species, *M. pumila*. Although relationships within the clades '*M. acicularis* – *M. madida*' and '*M. ferdinandiana* – *M. neowiedii*' were inconclusive, we propose the recognition of a single species, *M. acicularis,* for the former and of two species, *M. ferdinandiana* and *M. neowiedii,* for the latter based on morphological evidence. Nevertheless, more detailed phylogenetic studies are necessary to clarify diversification patterns and character evolution within these two clades. Deeper relationships were incongruent between the nuclear and chloroplast genomes. Cytogenetic data defined two groups, according to chromosome number and the number of CMA/DAPI bands: 2n=38, with more CMA^+ bands and 2n=36, with fewer CMA^+ bands. Banding patterns and chromosome counting obtained for *M. ferdinandiana* suggest the state of 2n=38 is ancestral for the *'Maxillaria madida'* complex, with 2n=36 species having experienced descendent dysploidy. Complementary studies considering more taxa as well as data from different molecular markers are already being developed in order to better understand diversification patterns within this group.

Resumo

Diferentes propostas para delimitação de espécies do complexo *'Maxillaria madida'* têm sido sugeridas ao longo da história devido, principalmente, à grande variabilidade de caracteres morfológicos e à plasticidade fenotípica. Com o objetivo de esclarecer limites de espécies e propor uma classificação mais estável para este grupo, este estudo tem por objetivo inferir relações filogenéticas considerando toda a variação morfológica e geográfica do grupo, obter dados de citogenética (contagem cromossômica e padrões de distribuição de heterocromatina) e descrever conjuntos de caracteres morfológicos diagnósticos que permitam a identificação de espécies. Os estudos filogenéticos basearam-se em dados de seqüências de DNA de núcleo (ITS1-2) e cloroplasto (espaçador *atpB-rbcL*, *trnL-F*, *matK* + intron *trnK*) de 51 amostras, utilizando os critérios de parcimônia máxima e verossimilhança máxima. Números cromossômicos e padrões de distribuição de heterocromatina baseados em CMA/DAPI foram obtidos para oito táxons. É proposto o reconhecimento de nove espécies para o complexo '*Maxillaria madida*'. Seis espécies atualmente reconhecidas foram suportadas pelos dados aqui obtidos. Além disso, caracteres morfológicos e moleculares sugerem que o grupo '*M. pumila*' deve ser reconhecido como uma única espécie, *M. pumila*. Relações filogenéticas dentro dos clados '*M. acicularis – M. madida*' e '*M. ferdinandiana – M. neowiedii*' foram inconclusivas e demandam estudos adicionais. De acordo com caracteres morfológicos diagnósticos é proposto o reconhecimento de uma única espécie para o clado '*M. acicularis – M. madida*' (*M.* acicularis) e de duas espécies para o clado '*M. ferdinandiana – M. neowiedii*'. Relações filogenéticas entre as espécies reconhecidas neste estudo mostraram-se incongruentes para os dados de cloroplasto e núcleo. Os dados de citogenética suportam dois grupos: 2n=38, com mais bandas CMA^+ e 2n=36, com um número de bandas CMA^+ reduzido. Padrões de distribuição de heterocromatina e número cromossômico obtidos para *M. ferdinandiana* sugerem que o estado 2n=38 é ancestral para o complexo *'Maxillaria madida'* e que as espécies com 2n=36 teriam sofrido disploidia descendente. Estudos complementares, considerando mais táxons e marcadores moleculares adicionais estão sendo desenvolvidos para uma melhor compreensão dos padrões de diversificação complexo '*Maxillaria madida*'.

Introduction

The subtribe Maxillariinae (sensu Whitten *et al*., 2000) comprises a monophyletic group of neotropical orchids with approximately 600–700 species, which can be generally distinguished by the presence of a distinct column foot and mentum, four rounded or ovoid pollinia, and a broad, open stigma (Whitten *et al*., 2000). Although Maxillariinae orchids comprise a major component of the epiphytic vegetation in the Neotropics, particularly the large and diverse genus *Maxillaria*, they are still taxonomically poorly known. For most species complexes within *Maxillaria* there is no consensus on how many species should be recognized. In addition, available keys for species identification are incomplete and subgeneric classifications are highly artificial. For this reason, phylogenetic studies based on sequence data are currently being developed in an attempt to define generic and subgeneric boundaries within this subtribe (Dathe and Dietrich, 2006; Whitten *et al*., unpubl. res.; www.flmnh.ufl.edu/natsci/herbarium/max/).

DNA sequence data from multiple regions strongly support a group of 25–34 taxa within *Maxillaria* as monophyletic (Whitten *et al*., unpubl. res.). Most species belonging to this group, denominated here as the '*Maxillaria madida*' complex, are restricted to south–eastern South America, ranging from the south of Bahia State, in Brazil, to Misiones, in Argentina. A few other species, however, are restricted to Central America and western South America, occurring from Bolivia up to southern Mexico. Although never recognized as a natural group, possibly because of such a disjunctive geographical distribution, species belonging to this group comprise small orchids that can be easily recognized by their coriaceous, thick to fleshy leaves, whitish to yellow or red to tan coloured flowers, generally with a shiny spot in the midlobe lip and, by the roots with annular expansions.

While the '*Maxillaria madida*' complex is easily distinguishable from other clades of Maxillariinae, species identification within it can be very challenging. Species concepts related to the '*Maxillaria madida*' complex, especially concerning the south–eastern Brazilian species, have varied largely among different taxonomic treatments, mainly because of the continuous nature of morphological characters used to differentiate taxa (Cogniaux, 1904; Hoehne, 1953; Pabst and Dungs, 1977; Butzin and Senghas, 1996). Table 1 summarizes the view of different authors on species delimitation within the '*Maxillaria madida*' complex.

In the first taxonomic treatment of the genus *Maxillaria* in Brazil, Cogniaux (1904) recognized 19 taxa (17 species and two new varieties) for the '*Maxillaria madida*' complex. Later, in several publications concerning the taxonomy of Brazilian species of *Maxillaria*, Hoehne (1947; 1952; 1953) described several new taxa, recognizing 31 taxa for the '*Maxillaria madida*' complex in his treatment for the Flora Brasilica (Hoehne, 1953). The diagnostic characters considered important by Hoehne (1953) to identify species within this group were number and shape of leaves, shape of perianth segments, and lip morphology (Table 1). Although the taxonomic descriptions published by Hoehne (1953) were remarkably detailed, clarity of species delimitation was hampered by the restricted number of specimens available for study as well by the difficulty in having access to type material.

In their classification of Brazilian orchids, Pabst and Dungs (1977) divided the species of *Maxillaria* in several alliances, according, primarily, to vegetative traits. The authors distributed the species of the '*Maxillaria madida*' complex in four different alliances, according to the number and shape of leaves. The only exception was *Maxillaria uncata*, which was assigned to a distinct alliance based on the presence of a long rhizome and an undivided lip (Pabst and Dungs, 1977).

In the last edition of Schlechter's "Die Orchideen" (Butzin and Senghas, 1996), the nine recognized species of the '*Maxillaria madida*' complex were divided into three main groups, according to leaf shape and plant architecture. The placement of the species *M. madida* in two different groups *per se* indicates the high infraspecific polymorphism of vegetative characters used for assigning group membership and the difficulty of classifying taxa within this clade based solely on morphological data. Interestingly, Schlechter was the only author to consider *M. uncata* (which occurs from northern South America to Mexico) in the same group as other species of the '*Maxillaria madida*' complex (restricted to southeast South America) based on the presence of fleshy leaves, erect pseudobulbs with brownish papery bracts and medium size, partially closed flowers, varying from yellowish to brownish.

Table 1. Former classifications proposed for the *'Maxillaria madida'* complex.

Author	**number of taxa recognized**	**Main diagnostic morphological characters**
Cogniaux (1904)	17 spp. and 2 varieties	not presented
Hoehne (1953)	31 spp.	number and shape of leaves, shape of perianth segments, lip morphology
Pabst & Dungs (1977)	23 spp.; 4 alliances: '*M. madida*', '*M. paulistana*', '*M. pumila*', '*M. subulata*'	number and shape of leaves
Butzin and Senghas (1996)	9 spp.; 3 groups: 'Nadelblättrige', 'Dickblättrige', 'aufsteigend Kletternde'	plant architecture and leaf shape

It is clear that the available taxonomic treatments failed to provide clear boundaries for species within the *'Maxillaria madida'* complex, possibly because of the continuous variable nature of morphological characters among current species concepts, especially plant architecture, leaf shape and lip morphology, as well due to highly polymorphic populations (Cogniaux, 1904; Hoehne, 1953; Pabst and Dungs, 1977; Butzin and Senghas, 1996). Some species belonging to this group also present extremely high phenotypic plasticity of vegetative characters, as observed for some cultivated specimens, probably related to different light conditions and humidity levels (S. Koehler, pers. obs.). Such extreme morphological variation within and among putative species and infraspecific taxa resulted in a plethora of species and infra–specific taxa with blurred boundaries.

Further investigation on morphological characters in the *'Maxillaria madida'* complex should remain an important source of information appropriate for species delimitation. Nevertheless, several studies dealing with morphologically complex groups of plants, including orchids, have also successfully used combined sequence data to establish species boundaries (e.g. Parker and Koopowitz, 1993). Species–level phylogenies are important not only to propose more stable, objective classifications for complex groups, but also to

assist the understanding of patterns of diversification and morphological evolution within such groups.

Despite being one of the largest genera of Orchidaceae, cytogenetic data on *Maxillaria* species are scarce and, therefore, chromosome evolution within this genus also remains poorly understood. Chromosome numbers of only nine species are currently known (Felix and Guerra, 2000; Cabral *et al.*, 2006), representing 0.02% of the species diversity of *Maxillaria*. Within the subtribe Maxillariinae, chromosome numbers of 2n = 38, 40, 42 are reported for *Maxillaria* and *Trigonidium*, with only one record for the *'Maxillaria madida'* complex (Dressler, 1993; Brandham, 1999; Felix and Guerra, 2000; Cabral *et al.* 2006). Based on chromosome data available for the subtribe Oncidiinae, Felix and Guerra (2000) suggested a base chromosome number of n = 20 for Maxillariinae, possibly derived by descending dysploidy from a hexaploid lineage with n = 21 (x = 7).

Besides the variation on chromosome numbers, another important source of information in cytogenetics is the study of heterochromatin distribution in blocks or bands throughout the karyotype (Guerra, 2000). Although information on heterochromatin distribution was never applied to systematic studies in orchids, the comparison of distinct heterochromatic patterns among species have proved to be a valuable tool in species delimitation as well in the identification of putative hybrids in other families, such as Rutaceae (Cornelio *et al.*, 2003) and Plantaginaceae (Martínez–Ortega *et al.*, 2004). Fluorochrome staining comprises a simple, reproducible method to identify patterns of variation of heterochromatic bands. The fluorochromes chromomycin A (CMA) and 4',6–diamidino–2–phenylindole (DAPI) exhibit preferential staining for GC and AT–rich DNA sequences, respectively, allowing the identification of different types of heterochromatin that may be used as chromosome markers (Guerra, 2000).

The main goals of this study were to understand patterns of diversification within the *'Maxillaria madida'* complex in order to assess the problem of species limits and to provide a more stable classification for this group. To achieve such aims, we proposed (1) to infer phylogenetic relationships within this group based on sequence data from six DNA regions (plastid *trnL* intron, *trnL–F* intergenic spacer, *matK*+*trnK* intron, and nuclear ribosomal internal transcribed spacers [ITS1–2] DNA regions and the 5.8S gene; (2) to provide data on the cytological diversity of the *'Maxillaria madida'* complex based on

chromosome counts and heterochromatin patterns, verifying its applicability on species delimitation in Maxillariinae orchids; and (3) to describe sets of diagnostic morphological characters that can be used for species identification according to the diversification patterns here obtained.

Materials and Methods

As previously stated, the monophyly of the '*Maxillaria madida*' complex has already been assessed by preliminary phylogenetic analyses based on sequence data considering 354 species of the subtribe Maxillariinae, including all specimens of the '*Maxillaria madida*' complex here sampled (Whitten *et al.*, unpubl. res.). Therefore, only two outgroup species, *Maxillaria lindleyana* and *M. ochroleuca*, were included in this study for tree rooting purposes. All samples were vouchered by herbarium specimens, including the ones used for cytogenetic studies (Tables 2 and 3). Samples *Koehler 73*, *Koehler 79*, *Koehler 91* and *Koehler 240* were excluded from the ITS matrix as were samples *Koehler 173*, *Koehler 243* and *Whitten 951* from the plastid matrix due to the poor quality of the data. We followed species criteria of Hoehne (1953) for preliminary identification and discussion of species limits within the '*Maxillaria madida*' complex , since it comprises the most recent taxonomic treatment available for the majority of species within the '*Maxillaria madida*' complex.

Laboratory protocols

DNA was extracted from fresh plant tissues (leaves and flowers) according to Doyle and Doyle (1987) and scaled down to 1 ml extraction volumes following the protocol described in Whitten *et al.* (2000), except that all samples were purified with QIAquick columns (Qiagen Inc., Valencia, California, USA) prior to amplification. Amplification was performed using 25–50 µl reactions, 2.5 mM $MgCl_2$, and a 10 min hot start, using Sigma (Sigma Inc., St. Louis, Missouri, USA) buffers and Taq polymerase. In all ITS samples, betaine (Sigma Inc.) was added (1.0 mM final concentration) to the PCR mix to relax secondary structure. Amplification and sequencing primers used for ITS and *trnL–F* regions were designed by Sun *et al.* (1994) and Taberlet *et al.* (1991), respectively.

Table 2. DNA vouchers for molecular phylogenetic studies of the '*Maxillaria madida*' complex.

Species	Localidade	Voucher info
M. acicularis Herb. ex Lindl.	Bahia, Brazil	*Koehler* 115 (SP)
M. acicularis Herb. ex Lindl.	Brazil	*Koehler* 237 (UEC)
M. acicularis Herb. ex Lindl.	Sooretama, Espírito Santo, Brazil	*Koehler* 345 (UEC)
M. acicularis Herb. ex Lindl.	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 371 (ESA)
M. acicularis Herb. ex Lindl.	Brazil	*Whitten 1994* (FLAS)
M. cogniauxiana Hoehne	Brazil	*Koehler* 240 (UEC)
M. echiniphyta Barb. Rodr.	Brazil	*Koehler* 353 (UEC)
M. echiniphyta Barb. Rodr.	Brazil	*Whitten* 1056 (FLAS)
M. echiniphyta Barb. Rodr.	Brazil	*Whitten 951* (FLAS)
M. ferdinandiana Barb. Rodr.	Camanducaia, Minas Gerais, Brazil	*Koehler* 109 (SP)
M. ferdinandiana Barb. Rodr.	Orleans, Santa Catarina, Brazil	*Koehler* 89 (SP)
M. heterophylla var. *acicularifolia* Hoehne	Cotia, São Paulo, Brazil	*Koehler* 1706 (SP)
M. heterophylla var. *acicularifolia* Hoehne	Itaperaí, São Paulo, Brazil	*Koehler* 95 (SP)
M. heterophylla var. *magnifolia* Hoehne	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 245 (UEC)
M. heterophylla var. *pygmaea* Hoehne	Cotia, São Paulo, Brazil	*Koehler* 113 (SP)
M. heterophylla var. *pygmaea* Hoehne	Serra do Cipó, Sanatana do Riacho, Minas Gerais	*Koehler* 278 (ESA)
M. heterophylla var. *pygmaea* Hoehne	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 292 (ESA)
M. juergensii Schltr.	Brazil	*Koehler* 111 (SP)
M. juergensii Schltr.	Itaperaí, São Paulo, Brazil	*Koehler* 69 (SP)
M. juergensii Schltr.	Brazil	*Koehler* 79 (SP)
M. lindleyana Schltr.	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 5 (UEC)
M. madida Lindl.	Petrópolis, Rio de Janeiro, Brazil	*Koehler* 107 (SP)
M. madida Lindl.	Brazil	*Koehler* 173 (SP)
M. madida Lindl.	Cunha-Parati, São Paulo, Brazil	*Koehler* 65 (SP)
M. madida Lindl.	Paranapiacaba, Santo André, São Paulo, Brazil	*Koehler* 81 (SP)
M. minuta Cogn	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 243 (UEC)
M. minuta Cogn.	Sooretama, Espírito Santo, Brazil	*Koehler* 253 (UEC)
M. minuta Cogn.	Brazil	*Koehler* 97 (SP)
M. mosenii var. *echinochila* Hoehne	Presidente Jucelino, Minas Gerais, Brazil	*Koehler* 294 (ESA)
M. mosenii var. *echinochila* Hoehne	Serra do Cipó, Santana do Riacho, Minas Gerais, Brazil	*Koehler* 87 (SP)
M. mosenii var. *hatschbachii* Hoehne	Cananéia, São Paulo, Brazil	*Koehler* 71 (UEC)

Species	Localidade	Voucher info
M. mosenii var. *hatschbachii* Hoehne	Paranapiacaba, Santo André, São Paulo, Brazil	*Koehler* 83 (SP)
M. nardoides Kraenzl.	Ecuador	*Whitten* 2359 (FLAS)
M. nardoides Kraenzl.	Ecuador	*Whitten* 2502 (FLAS)
M. neowiedii Rchb.f.	Cunha-Parati, São Paulo, Brazil	*Koehler* 73 (SP)
M. neowiedii Rchb.f.	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 91 (SP)
M. ochroleuca Lodd. ex Lindl.	I. Cardoso, Cananéia, São Paulo, Brazil	*Koehler* 11 (UEC)
M. pacholskii Schltr.	Ecuador	*Whitten* 2393 (FLAS)
M. pacholskii Schltr.	Ecuador	*Whitten* 2464 (FLAS)
M. pachyphylla Schltr. ex Hoehne	Santa Catarina, Brazil	*Koehler* 105 (SP)
M. pachyphylla Schltr. ex Hoehne	Hort ESA 8903	*Koehler* 369 (ESA)
M. plebeja Rchb.f.	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 1653 (SP)
M. plebeja Rchb.f.	Bertioga, São Paulo, Brazil	*Koehler* 85 (SP)
M. pumila Hook.	Peruíbe, São Paulo, Brazil	*Koehler* 101 (SP)
M. pumila Hook.	Brazil	*Koehler* 355 (UEC)
M. pumila Hook.	Brazil	*Koehler* 94 (SP)
M. uncata Lindl.	Brazil	*Koehler* 359 (UEC)
M. uncata Lindl.	Brazil	*Koehler* 75 (SP)
M. uncata Lindl.	Ecuador	*Whitten* 2394 (UEC)
M. uncata Lindl.	Ecuador	*Whitten* 2394 (FLAS)
M. vernicosa Barb. Rodr.	Castro Alves, Bahia, Brazil	*Koehler* 103 (SP)
M. vernicosa Barb. Rodr.	Caldas, Minas Gerais, Brazil	*Koehler* 99 (SP)
Maxillaria sp.	Peru, Cult.	*Whitten* 2310 (FLAS)

Table 3. Species of the *'Maxillaria madida'* complex sampled for cytogenetic studies, with respective locality, voucher number, chromosome number, predominant type of chromosomes (meta and m = metacentric, sub and sm = submetacentric, acro and a = acrocentric, t = terminal, st = subterminal, I = interstitial) and pattern of DAPI+ and CMA+ bands.

Species	**Locality**	**Voucher info**	**2n**	**Karyotype**	**DAPI$^+$ (2n)**	**CMA$^+$ (2n)**
Maxillaria ferdinandiana Barb. Rodr.	Cult., Brazil	*Koehler* C1 (UEC)	36	acro	4m	2t
M. heterophylla var. *pygmaea* Hoehne	Cult., Brazil	*Koehler* C2 (UEC)	36	meta/acro	10m-sm + 6a	2t
M. pachyphylla Schltr. ex Hoehne	Cult., Brazil	*Koehler* C3 (UEC)	36	sub/acro	2sm + 6a	2t
M. pumila Hook.	Cult., Brazil	*Koehler* C4 (UEC)	36	acro	2m + 10a	2t + 1i
M. acicularis Lindl.	Cult., Brazil	*Koehler* C5 (UEC)	38	acro	20a	3st + 2t
M. acicularis Lindl.	Floresta Azul, Bahia, Brazil	*Koehler* 17744 (ESA)	38	acro	20a	3st + 2t
M. acicularis Lindl.	Nova Friburgo, Rio de Janeiro, Brazil	*Koehler* 23759 (ESA)	38	acro	16a	4st + 2t
M. mosenii var. *echinochila* Hoehne	Caeté, Minas Gerais, Brazil	*Koehler* C6 (UEC)	76	acro	30-32a	6t
M. mosenii var. *echinochila* Hoehne	São Fidelis, Rio de Janeiro, Brazil	*Koehler* 15142 (ESA)	76	acro	30-32a	7t
M. mosenii var. *echinochila* Hoehne	Domingos Martins, Espítiro Santo, Brazil	*Koehler* 31932(ESA)	38	acro	18-20a	2st + 6t
M. madida var. *monophylla*	Santa Maria do Salto, Minas Gerais, Brazil	*Custódio* C7 (UEC)	38	acro	18a	2st + 5t
M. madida var. *monophylla* Cogn.	Una, Bahia, Brazil	*Koehler* 18780 (ESA)	38	acro	20a	2st + 4t
M. madida var. *monophylla* Cogn.	Santa Luzia, Bahia, Brazil	*Koehler* 19110 (ESA)	38	acro	20a	2st + 4t
M. madida Lindl.	Jacareí-Ribeirão Claro, São Paulo, Brazil	*Koehler* 13588 (ESA)	38	acro	14a	1st + 3t
M. madida Lindl.	Santo André, São Paulo, Brazil	*Koehler* C8-12000 (SP)	38	acro	14a	1st + 2t
M. madida Lindl.	São Miguel Arcanjo, São Paulo, Brazil	*Koehler* 33428 (ESA)	38	acro	14a	1st + 3t
M. madida Lindl.	Diamantina, Minas Gerais, Brazil	*Koehler* 33084 (ESA)	38	acro	14a	1st + 3t

Some amplification reactions for *trnL–F* using primers C and F produced multiple bands – for these samples, reactions were performed using also D and E primers. For the amplification of *matK* +*trn*K intron we used primers 19F (Goldman et al. 2001), trnK2R (Johnson and Soltis 1994) and two additional internal sequencing primers specifically designed by M. Whitten for Maxillariinae orchids 5′- CAT TTC TAA TAA ATA CTC TGA C-3′ [1100R] and 5′-TAT CAG AAG GTT TTG (CG)A-3′ [308F]. Primers for amplification of the *atpB–rbcL* spacer region were also designed by M. Whitten, 5′-AGA AGT AGT GGG ATT GCT TCT C-3′ [Max F], 5′-TCA CAA CAA CAA GGT CTA CTC G-3′ [Max R]). Protocols for the amplification reactions were as follows: [ITS] ten min bake at 99 °C , 30 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s, 72 °C extension for 1 min; [*trnL-F*] 32 cycles of 94 °C denaturation for 30 s, 61 °C annealing for 30 s, 72 °C extension for 75 s; [*mat*K and atpB-rbcL spacer] 33 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s, 72 °C extension for 2 min. Amplified products were purified with QIAquick PCR cleaning column and filtration kit (Qiagen Inc.) and directly sequenced on Applied Biosystems, Inc (ABI) 373/377 or 3100/3500 automated sequencers using standard dye–terminator according to manufacturer's protocols, except that cycle sequencing reactions were scaled down to 5µl. Both strands were sequenced to assure accuracy in base calling.

Data Analysis

Alignment. The software packages "Sequence Navigator™", "Autoassembler™" (ABI) and "Sequencher™" (Gene Codes Corporation) were used to edit and assemble complementary and overlapping sequences. Each individual base position was examined for agreement of the two strands. DNA sequences were aligned manually, and gaps were coded as missing values. Terminal priming regions were excluded, as were regions where alignment was ambiguous or where extensive length variation occurred. Aligned matrices are available upon request.

Molecular data exploration. The null hypothesis of base frequency stationary among sequences was evaluated using the chi-square heterogeneity test as implemented in PAUP* 4.0b10 (Swofford, 2000). The g1 statistic was used to determine if phylogenetic signal was significantly non–random (Hillis and Huelsenbeck, 1992). The left skew of tree

distributions was obtained in PAUP* based on 10,000 randomly generated trees. The g1 statistic was then compared to the significance levels presented by Hillis and Huelsenbeck (1992). Also, possible incongruence between nuclear and chloroplast genomes was assessed with the incongruence length difference (ILD) test (Farris *et al.*, 1994), implemented in PAUP* as the partition homogeneity test using 1000 replicates and excluding uninformative characters to avoid overestimation of the amount of incongruence (Lee, 2001).

Phylogenetic analyses. Phylogenetic analyses were initially conducted with a heuristic search under the maximum parsimony (MP) criterion of Fitch (unordered characters, equal weights to all changes; Fitch, 1971), excluding uninformative characters, and with ACCTRAN optimization. Trees were reconstructed both with and without outgroups to check for possible long–branch attraction artefacts (Bergsten, 2005). The search strategy for all data sets used 10,000 addition sequence replicates by stepwise addition holding 10 trees/replicate, TBR branch swapping on best trees, MULTREES on, saving no more than 10 optimal trees with score = 100 over each replicate. To assess the internal support of internal clades we performed 1000 bootstrap pseudo–replicates (Felsenstein, 1985) of 10 addition sequence replicates by stepwise addition holding 1 trees/replicate. The categories of bootstrap support considered in this study were: unsupported (<50%); weak (50–74%); moderate (75–84%); strong (85–100%) (Whitten *et al.*, 2000). In order to decrease the effect of possible random noise in the tree estimation process, we also applied the successive weighting strategy (SW) for maximum parsimony analyses (Farris, 1969; Carpenter, 1994). Optimization of successive weighting analyses were carried out considering 1000 addition sequence replicates and SPR branch swapping with characters being reweighed according to the rescaled index until tree scores were not improved. Then, a final analysis considering the same search strategy applied for the unweighted data was conducted (10,000 addition sequence replicates, TBR branch swapping).

Maximum likelihood analyses (ML) were also carried out. First, alternative nested models of DNA sequence evolution were evaluated with likelihood ratio tests as implemented in MODELTEST 3.7 (Posada and Crandall, 1998; $\alpha = 0.01$). The best–fit model of DNA sequence evolution with its estimated parameters was then input into

detailed maximum–likelihood tree searches performed in PAUP*. Starting trees were obtained using 10 addition sequence replicates by stepwise addition holding 1 tree/replicate, with further SPR branch swapping. Starting branch–lengths were obtained using the Rogers–Swofford approximation method with branch–length optimization of Newton–Raphson. Confidence on the obtained ML trees was assessed by bootstrap analyses based on 100 pseudo–replicates using the fast reduced search option in PAUP*.

Cytogenetic studies

Root tips were collected and pre–treated in 0.002 M 8–hydroxyquinoline for 20 h at 8 ^{o}C. Samples were then fixed in Carnoy (ethanol : acetic acid : 3:1) for 2 h at room temperature and stored at –20 ^{o}C. Root tips were digested with 2% cellulose / 20% pectinase for 90 min at 37 ^{o}C. The meristem was subsequently isolated and squashed in 45% acetic acid. After coverslip removal the slides were aged for 3 days at room temperature. The aged slides were double stained according to Schweizer and Ambros (1994) with CMA (0.5 mg mL^{-1}, 1 h) and DAPI (2 µg mL^{-1}, 30 min), and mounted in McIlvaine's (pH 7.0) buffer–glycerol (v/v/ 1:1) containing 2.5 mM $MgCl_2$. After 3 days of fluorochrome stabilization, the best slides were analyzed in a Leica DMLB microscope and cell images were captured with a COHU CCD video camera using the QFISH software (Leica).

Results

Phylogenetic analyses

The test for phylogenetic signal based on random–tree distributions showed that the all data sets contain significant phylogenetic information (g1 values = –0.40 [ITS]; –0.55 [plastid]; p<= 0.0014). We were unable to detect any significant heterogeneity in base frequencies among taxa using the chi–square heterogeneity test for the ITS data set (p= 0.99). However, the chi–square test rejected the hypothesis of base frequency homogeneity for the plastid data set (p= 0.004). The null hypothesis of congruence between the nuclear and plastid data sets was strongly rejected by the ILD test (p= 0.001). As visual inspection

of nuclear and plastid topologies also clearly indicated they are significantly discordant, no combined analysis was conducted.

Tree statistics of MP and SW analyses are summarized in Table 4. Bootstrap analyses were conducted only for unweighted data. No topological differences were observed between rooted and unrooted (outgroup excluded) trees, what eliminates the possibility that long branch attraction artefacts are caused by longer branches of outgroup taxa (Bergsten, 2005).

ITS nrDNA. The MP and ML statistics for the ITS analysis are indicated in Tables 4 and 5, respectively. Five morphologically cohesive clades are strongly supported by ML, MP and SW analyses (Fig. 1). These correspond to the currently recognized species *Maxillaria echiniphyta* (Fig. 1E), *M. nardoides* (Fig. 1B), *M. pachyphylla* (Fig. 1F), *M. uncata* (Fig. 1G) and *M. vernicosa* (Fig. 1H). Two species groups, currently composed by species with extremely similar morphology also emerged as monophyletic groups from this analysis: the *'M. acicularis – M. madida' – M. mosenii* clade and the *M. minuta – M. plebeja – M. pumila* clade - these are denominated here as *'M. acicularis – M. madida'* (Fig. 1A) and '*M. pumila*' (Fig. 1D), respectively. The clade *M. cogniauxiana – M. ferdinandiana – M. heterophylla – M. juergensii – M. neowiedii* (= '*M. ferdinandiana – M. neowiedii*' clade; Fig. 1C), comprises two morphologically distinct groups, the sampled specimens of *M. ferdinandiana* and the *M. cogniauxiana – M. heterophylla – M. juergensii – M. neowiedii* group. The currently accepted species *M. pacholskii* also appears as a monophyletic group in the ITS analysis, but with weak bootstrap support (Fig. 1I). Other strongly supported clades obtained from both MP and ML analyses of ITS data were (1) the *'M. acicularis – M. madida'* clade + *M. nardoides* + *Maxillaria* sp.; (2) *M. uncata*, *M. vernicosa* and *M. pacholskii*; and (3) *M. echiniphyta*, *M. pachyphylla*, the '*M. pumila*' clade, and the '*M. ferdinandiana – M. neowiedii*' clade (Fig. 1). Two clades were only recovered in the ML and SW analyses: ('*M. acicularis – M. madida*' clade, *M. nardoides*) and (('*M. ferdinandiana – M. neowiedii*' clade, (*M. echiniphyta*, '*M. pumila*' clade) *M. pachyphylla*) (*M. uncata*, *M. vernicosa* and *M. pacholskii*)) (Fig. 1).

Table 4. Statistics from phylogenetic analyses performed under the maximum parsimony criterion. (*) Results under successive weighting strategy.

Data set	# ingroup taxa	chi-square homogeneity test results	g1 statistics	total no characters (informative)	MPT (*)	length (*)	CI(*)	RI(*)	RC
ITS	49	91.19 (df=150, p= 0.99)	-0.40	748 (14.4%)	1440 (260)	166 (127.3)	0.80 (0.90)	0.95 (0.98)	0.88
plastid	48	189.96 (df = 141, p= 0.004)	-0.55	4235 (6.3%)	39,366 (2,257)	454 (232.9)	0.65 (0.88)	0.87 (0.97)	0.85

Table 5. Statistics from phylogenetic analyses performed under the maximum likelihood criterion.

Data set	# ingroup taxa	Selected model	Nucleotide frequencies	shape parameter [α value] of gamma-distributed rate variation across sites	Pinvar	-lnL value
ITS	49	General Time Reversible	A= 0.21, C= 0.28, G= 0.32, T=0.19	0.47	NA	2528.53
plastid	46	F81+G+I	A= 0.32, C= 0.15, G= 0.14, T=0.39	0.90	0.61	10986.45

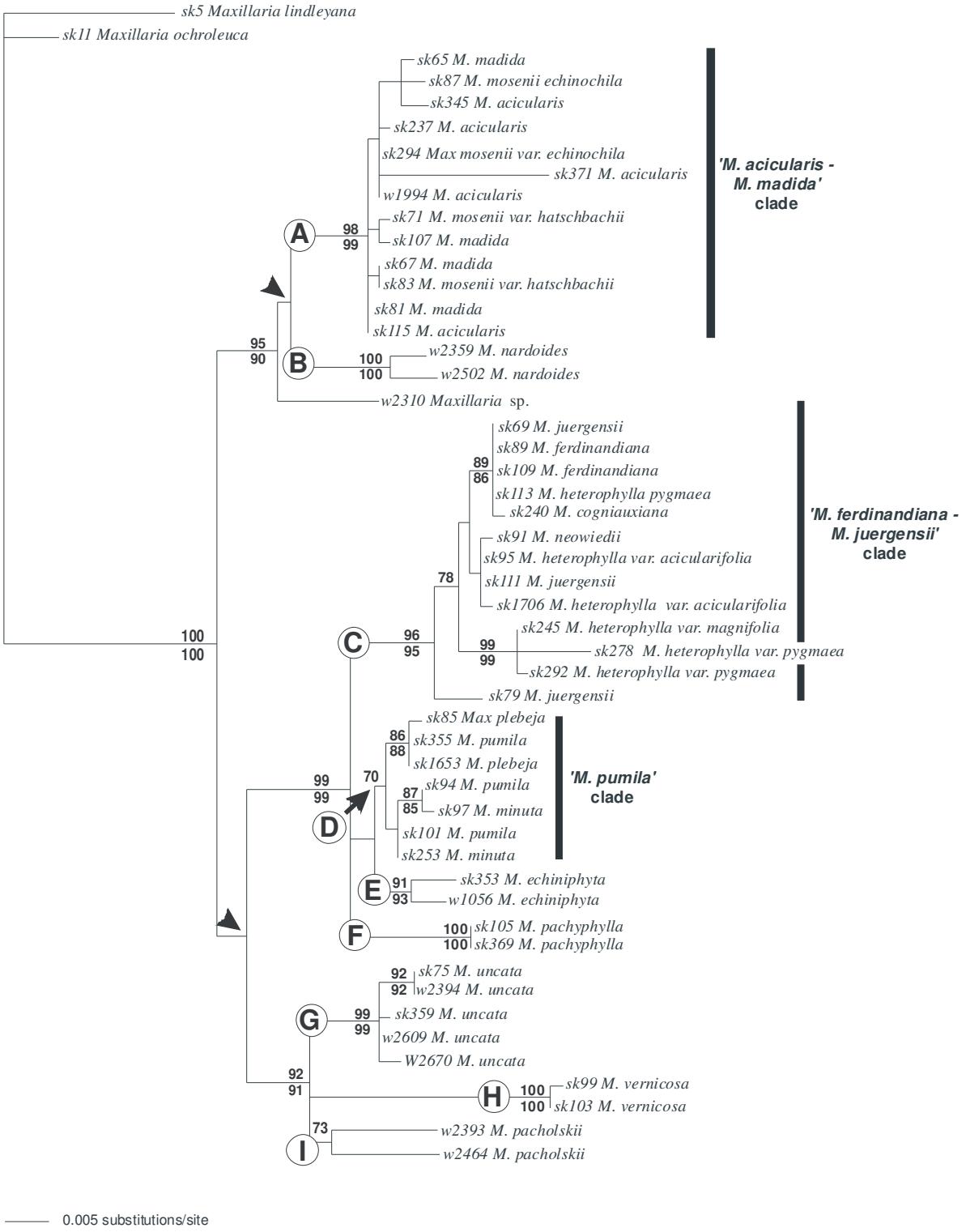


Fig. 1. Maximum likelihood phylogram (-lnL= 2528.53) for ITS nrDNA data. Maximum likelihood/maximum parsimony bootstrap support values are indicated above/below branches. Nodes that received less than 70% support are not marked. Nodes not supported in the strict consensus maximum parsimony tree are indicated by arrows.

Plastid [5]**.** The MP and ML statistics for the plastid analysis are indicated in Tables 4 and 5, respectively. Several clades with bootstrap support greater than 80% in common with the ITS data set was recovered by MP, SW and ML analyses of plastid data (Fig. 2): the '*M. acicularis – M. madida*' (Fig. 2A) and the '*M. pumila*' (Fig. 2D) clades, *M. echiniphyta* (Fig. 2E), *M. vernicosa* (Fig. 2H), *M. pacholskii* (Fig. 2I) and *M. uncata* (Fig. 2G). Although the clade '*M. ferdinandiana – M. neowiedii*' was also recovered by the MP, SW and ML strict consensus trees, it was not supported in the bootstrap consensus tree (Fig. 2C).

Despite the many terminal clades in common with the ITS data set, deeper nodes in the ML, MP and SW plastid trees were strongly incongruent with those based on ITS data (Figs. 1-2). The plastid trees did not support the clade '*M. uncata* + *M. vernicosa* + *M. pacholskii*' as sister to the clade '*M. pumila*' + '*M. juergensii – M. ferdinandiana*' + *M. echiniphyta* + *M. pachyphylla*. Instead, the latter is indicated as sister to the '*M. acicularis – M. madida*' clade, with *M. vernicosa* embedded in it, although none of these alternatives received bootstrap support greater than 50% (nodes collapsed). The plastid tree also supports *M. nardoides* as sister to *Maxillaria* sp. whereas ITS data supports the former as sister to the '*M. acicularis – M. madida*' clade. However, none of these clade received bootstrap support higher than 50% – MP analyses of plastid data did not even support *Maxillaria* sp. and *M. nardoides* as sister to the '*M. acicularis – M. madida*' clade (Fig. 2). Individual plastid trees (not shown) in general supported species and species groups recognized here but with a lot less resolution[5].

[5] Os resultados das análises filogenéticas considerando individualmente as regiões do genoma de cloroplasto são apresentadas no Anexo 3.

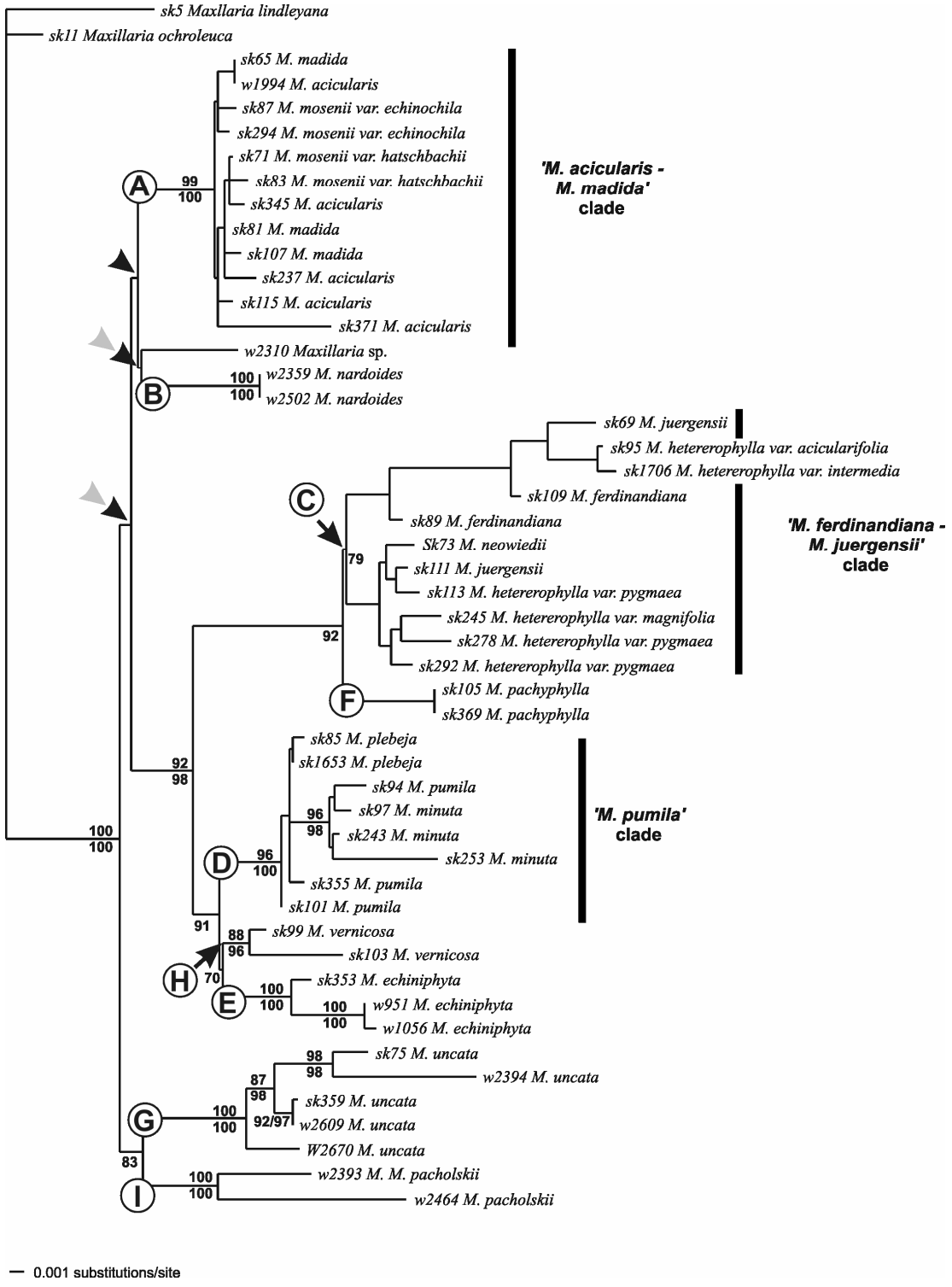


Fig. 2. Maximum likelihood phylogram (-lnL= 10986.45) for plastid data (*atpB-rbcL* spacer, *trnL-F*, *matK* regions). Maximum likelihood/maximum parsimony bootstrap support values are indicated above/below branches. Nodes that received less than 70% support are not marked. Nodes not supported in the strict consensus maximum parsimony are indicated by arrows (black arrows, unweighted analysis; grey arrows, weighted analysis).

Cytogenetic data for the species analyzed in the present study are summarized in Table 3. Variation in chromosome numbers defined two groups: 2n = 36 (*M. ferdinandiana, M. heterophylla, M. pachyphylla, M. pumila*) and 2n = 38 (*M. acicularis, M. madida, M. mosenii*). Polyploid specimens were observed in two samples of M. mosenii var. echinochila (2n = 76). All studied species revealed symmetric karyotypes with predominance of acrocentric chromosomes, except for *M. heterophylla* and *M. pachyphylla*, who also showed several metacentric and submetacentric chromosomes, respectively (Table 3).

There was considerable variability regarding number and localization of CMA^+ bands (brighter bands for CMA and duller for DAPI) and $DAPI^+$ (Table 3), appropriate for discriminating all the analysed species and infra-specific taxa. CMA^+ bands varied in number and were mainly terminal or subterminal on the long arm of acrocentric chromosome pairs, while $DAPI^+$ bands varied in number but were always proximal to the centromere. Metaphases of the different banding patterns observed in the studied species are depicted in Fig. 3. Heteromorphism of CMA^+ bands, evident through the odd number of bands, was common in specimens of the '*M. acicularis – M. madida*' clade as well in the '*M. pumila*' clade. Variation in banding patterns also supported the same two groups characterized by distinct chromosome numbers. Species with 2n = 36 generally had fewer heterochromatic CMA^+ blocks and more interspecific variability in the number of $DAPI^+$ bands, although there were always $DAPI^+$ bands present in at least one meta– or submetacentric pair (Figs. 3A-D).

On the other hand, species with 2n = 38 (Figs. 3E - L) had more CMA^+ bands, less variation in number of $DAPI^+$ bands (never in metacentric pairs), and occurrence of both heterochromatin types in some chromosome pairs (Fig. 3H). Since preliminary studies on *M. uncata* indicated the occurrence of 2n=36 for this species (J. S. Cabral, pers. obs.), we expect that cytogenetic patterns of *M. vernicosa* would also agree with other species belonging to the 2n=36 group, despite the uncertainty of its phylogenetic position within the '*M. madida – M. uncata*' clade. The species *M. ferdinandiana*, also belonging to the 2n=36 group, was remarkable for bearing only one chromosome pair with duplicated $DAPI^+$ bands, presumably each one at one side of the centromere (Fig. 3A).

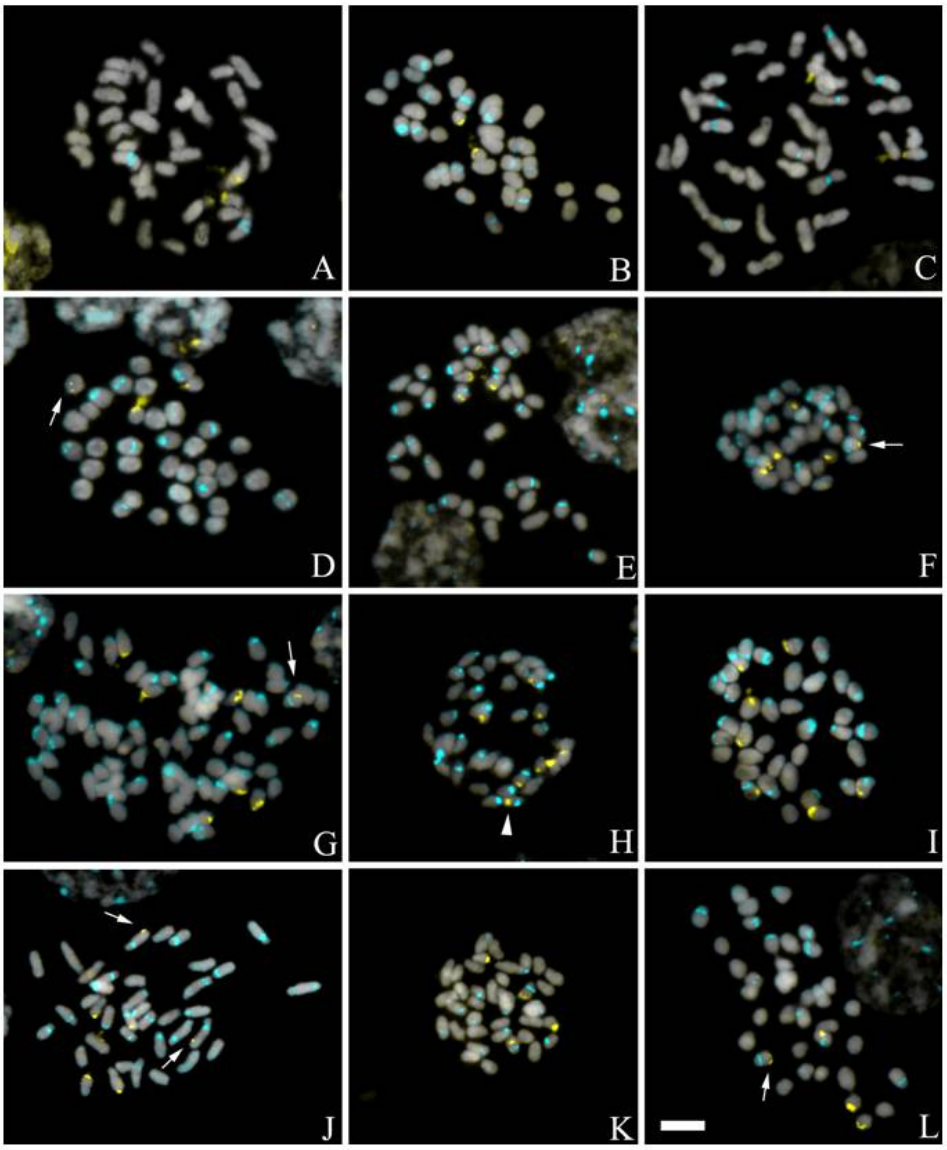


Fig. 3. Banding patterns of metaphase cells showing CMA⁺ bands (yellow) and DAPI⁺ bands (blue). *Maxillaria ferdinandiana* (A), *M. heterophylla* var. *pygmaea* (B), *M. pachyphylla* (C), *M. pumila* (D), *M. acicularis* (E – F), *M. mosenii* var. *echinochila* (G - H), *M. madida* var. *monophylla* (I - J) and *M. madida* (K - L). Note a chromosome pair with two proximal DAPI⁺ bands in A. Arrows indicate very small CMA⁺ bands. Arrowhead in H indicates an example of chromosome with both DAPI and CMA bands. The gray color in the photographs is due to the overlay of colors of both fluorochromes. Bar in L corresponds to 5 μm.

Discussion

Nuclear vs plastid incongruence

Incongruence among different data partitions, as suggested by the results of the PH T performed in this study comprises a rather complex subject in phylogenetic systematics which has received increasing attention over the last decade (Bull *et al.*, 1993; Huelsenbeck *et al.*, 1996; Cunningham, 1997; Reed and Sterling, 1999). Several studies have demonstrated that incongruence may be caused by distinct categories of bias, namely random and systematic errors and independent evolutionary histories of partitions (Swofford *et al.*, 1996; Reed and Sterling, 1999). Therefore, there is no single answer for how to deal with it. In this study, we attempted to reduce random error by broadly sampling taxa as well different genome regions. Sampling efforts were mainly centred on highly polymorphic species groups (the '*M. pumila*', '*M. ferdinandiana – M. neowiedii*' and '*M. acicularis – M. madida*' clades) and bootstrap analyses were performed to assess confidence on the obtained results. Despite sampling efforts, absence of sufficient phylogenetic signal is likely to be the problem in deep levels of the recovered trees. While the ITS trees (MP and ML) suggest the (*M. pacholskii* + *M. vernicosa* + *M. uncata*) clade to be sister to the (*M. pachyphylla* + *M. echiniphyta* + '*M. pumila*' + '*M. juergensii – M. ferdinandiana*') clade, the ML plastid topology indicates the latter (including *M. vernicosa*) as sister to the ('*M. acicularis – M. madida*' + *M. nardoides* + *Maxillaria* sp) clade. None of these alternatives, however, was supported by bootstrap values greater than 50% (Figs. 1-2). Soft incongruence seems also to be the reason of low resolution within and between terminal clades, especially concerning the '*M. juergensii – M. ferdinandiana*' clade, although there are enough diagnostic morphological characters that allow identification of such groups as distinct taxonomic entities (see discussion below).

Systematic error may be caused either by the non–independence of characters or homoplasy (Swofford *et al.*, 1996). One way of dealing with it is improving the reconstruction model for individual partitions (Cunningham, 1997), such as conducting analyses under the maximum likelihood criterion, as performed in this study. Since simulation experiments have shown that high levels of homoplasy can decrease the accuracy of phylogenetic inference under the parsimony criterion (Huelsenbeck and Hillis, 1993), we also employed the successive weighting strategy for parsimony analyses. Both

successive weighting and ML analyses clearly improved tree topology definition, especially in deeper nodes of the plastid tree (Figs. 1-2), which suggests the lower resolution of MP trees may be a result of homoplasy due to mutational saturation.

The results also suggest the occurrence of genealogical discordance concerning the position of *M. vernicosa*, since alternative placements of this species in nuclear and plastid trees under all search criteria both received high bootstrap values. Four additional MP analyses were performed excluding outgroups as well all samples of *M. vernicosa*, *M. echiniphyta* and *M. uncata* plus *M. pacholskii* to check for possible occurrence of long branch attraction artefacts, but the obtained trees did not result in any distinct topologies (results not shown).

Genealogical discordances among topologies based on the same set of organisms may be attributable to several biological processes, such as lineage sorting of ancestral polymorphisms, reticulation processes, as well haplotype polymorphism within and among individuals in a population (see Wolfe and Randle, 2004 for a review). Phylogenetic inference of recently evolved species may particularly suffer from incomplete lineage sorting, one of most common causes of phylogenetic discordance among different gene trees (Lyons-Weiler and Milinkovitch, 1997; Maddison and Knowles, 2006). All trees obtained in this study (nuclear and plastid genomes) presented several clades with short branch lengths between the most recent common ancestor node and the terminal branches, which suggests that recent diversification has occurred in the '*Maxillaria madida*' complex. Hybridization and introgression also comprise potential causes of incongruence among phylogenetic trees (Mansion *et al.* 2005; Buckley *et al.*, 2006). The occurrence of recent and rapid divergence of species, sympatric populations and generalist pollinators in this group (S. Koehler, unpubl. res.) certainly reinforces the likelihood of reticulation events. Presumed hybrid individuals from natural populations bearing intermediate phenotypes have been reported for the '*Maxillaria madida*' complex (Hoehne, 1953; Onishi, 1974), although such scenario remains to be demonstrated. Both lineage sorting and reticulation processes can result in similar phylogenetic patterns (Holder *et al.*, 2001) and further studies would be necessary to access the role of each process in the diversification of the '*Maxillaria madida*' complex .

It is also possible that the obtained plastid topologies may have been influenced by *trnL–trnF* paralogues determined by haplotype polymorphisms, since multiple copies of this region as well polymorphisms of sequence eletropherograms have been observed in some samples. According to Wolfe and Randle (2004), if the copy number per cell of paralogous loci is sufficient to allow PCR amplification, the probability that paralogues will be included in the analyses increases. However, analyses based on plastid markers excluding the *trnL–trnF* data set also placed *M. vernicosa* as closely related to *M. pachyphylla*, *M. echiniphyta* as well to the '*M. ferdinandiana* – *M. neowiedii*' and '*M. pumila*' clades.

Species delimitation [6]

Despite the fact the null hypothesis of congruence among topologies based on chloroplast and nuclear data sets was strongly rejected by the ILD test, there was consensus concerning delimitation of morphological entities within the '*Maxillaria madida*' complex. Our results support the current delimitations of the species *M. echiniphyta* (clade E), *M. pachyphylla* (clade F), *M. vernicosa* (clade H), *M. pacholskii* (clade I), *M. nardoides* (clade B) and *M. uncata* (clade G) (Figs. 1–2; Hoehne, 1953; Pabst and Dungs 1977), which are also easily characterized by sets of diagnostic morphological characters.

Within species from south–eastern Brazil, *M. echiniphyta* (clade E) and *M. vernicosa* (clade H) comprise plants up to 5 cm high with bifoliate pseudobulbs bearing needle–like leaves and flowers with pedicels always longer than the adjacent pseudobulb. *M. echiniphyta* has white–pinkish flowers with elongate segments, while *M. vernicosa* has yellow flowers with ovate–oblanceolate segments (Barbosa Rodrigues, 1996). Another currently recognized species from south–eastern Brazil corroborated by our results is *M. pachyphylla* (clade F), a species growing up to 25 cm. It is distinctive by its unifoliate pseudobulbs bearing a thick, lanceolate leaf and pale yellow flowers with pedicels shorter than the adjacent pseudobulbs.

Our results indicate that the Amazonian species included in the '*Maxillaria madida*' complex belong to two distinct clades (Figs. 1–2). One of them comprises *M. nardoides*

[6] Fotos das espécies aqui descritas são apresentadas no Anexo 5.

(clade B) and *Maxillaria* sp. (a new species from Ecuador). *M. nardoides* is easily recognized by its 3–4 needle-like leaves in each pseudobulb and brownish to red–purplish flowers with a short viscidium (Bennet and Christenson, 1993). *Maxillaria* sp. is morphologically very similar to some populations of *M. madida* (*M. madida* var. *monophylla* Cogn.), being a large plant with big, reddish flowers and unifoliate pseudobulbs. However, the analyses herein presented suggest this specimen could be a separate species.

The other Amazonian clade found within the '*Maxillaria madida*' complex includes *M. uncata* (clade G) and *M. pacholskii* (clade I) – two species indeed morphologically very similar, bearing unifoliate pseudobulbs and elongated flowers with an extremely long stipe (up to 25 mm). They are easily distinguished by the colour of flowers and leaf morphology. *M. pacholskii* can be recognized by its membranaceous, linear leaves and red–brownish flowers (Christenson, 2003), while the widespread *M. uncata*, although vegetatively highly polymorphic, is easily identified by its fleshy to coriaceous leaves and white to lavender flowers (Atwood and Mora de Retana, 1999). Morphological diversification within *M. uncata* certainly deserves further attention, and possibly two or more taxa are currently embedded in the current *M. uncata* species concept, as already observed by Atwood and Mora de Retana (1999).

Three clades also emerged as well supported groups of species. These are the '*M. pumila*' clade (clade D), the '*M. acicularis* – *M. madida*' clade (clade A) and the '*M. ferdinandiana* – *M. neowiedii*' clade (clade C) (Figs. 1-2). They all include species from south–eastern Brazil that have been shown to be very difficult to distinguish, since they are defined by continuously morphological characters. The '*M. pumila*' clade (clade D) currently comprises, at least, three species, as demonstrated by our analyses (*M. pumila*, *M. plebeja* and *M. minuta*). Hoehne (1953) distinguished them based on the size and shape of leaves and on the shape and colour of flowers, but overlapping of morphological diagnostic characters is evident as soon as one attempts to identify specimens within this group. In addition, such taxa are neither geographically nor ecologically isolated, being restricted to the humid and seasonally dry forests of south–eastern Brazil. Thus, morphological variation is not reflected by geographic distribution nor by habitat variation, what reinforces the recognition of a single, polymorphic species for this group, namely *M. pumila*. Despite being highly polymorphic, *M. pumila* can be easily

distinguished from other species of the '*Maxillaria madida*' complex by its cylindrical pseudobulbs bearing one coriaceous to fleshy leaf and small yellowish–red flowers with pedicels always shorter than the adjacent pseudobulb.

Current species limits within the '*M. acicularis* – *M. madida*' clade (clade A, Figs. 1–2) are also blurred by continuous morphological characters, usually concerning leaf shape and flower size. Plants within this clade can be distinguished from others of the '*Maxillaria madida*' complex as larger plants up to 30 cm height, generally with reddish–brown flowers always shorter than the adjacent pseudobulb. Sampled individuals in this group also presented chromosome numbers of 2n = 38 with generally more CMA^+ and $DAPI^+$ bands when compared to other sampled clades (Table 3, Fig. 3). Most flowers produce a strong fruity, watermelon, fragrance which is very distinctive. However, contrary the '*M. pumila*' clade, morphological variation within the '*M. acicularis* – *M. madida*' is restricted geographically and ecologically. *M. acicularis* has traditionally been considered as a more delicate species with bifoliate pseudobulbs, needle–like leaves and smaller flowers, being restricted to forested habitats of south–eastern Brazil, while *M. madida* and *M. mosenii* correspond to more robust plants with larger pseudobulbs and flowers, and with 1–2 leaves varying from linear–lanceolate to cylindrical. The occurrence of tetraploidy in two accessions of *M. mosenii* var. *echinochila* might be related to the lithophytic habit, as polyploidy may give special advantages in colonizing harsh environments (Stebbins, 1966). The event of autopolyploidization in the successful colonization of new, harsh habitats was also suggested for species of *Laelia* (Blumenschein, 1960), *Pleione* (Stergianou, 1989), *Oncidium*, *Catasetum* (Felix and Guerra, 2000) and *Anacamptis pyramidalis* (Del Prete *et al.*, 1991). Some populations of *M. madida* and *M. mosenii* also occur in wet and seasonally dry forests of south–eastern Brazil, while others are found only on rocky outcrops (Hoehne, 1953; Onishi, 1974). Species boundaries among *M. madida*, *M. mosenii* and *M. acicularis* have never been questioned, despite the fact there are, at least, six names currently available for this complex (S. Koehler, in prep.). Unfortunately, the sequence data here presented are not informative enough to allow elucidation of the patterns of morphological diversity within this clade, although CMA/DAPI banding patterns, in general, support ecological and geographical subdivisions within this group (Table 3, Fig.

3). Further phylogenetic studies, considering more informative molecular markers at population levels are necessary before any taxonomical change is proposed.

Another highly polymorphic clade that emerged from our analyses is the '*M. ferdinandiana – M. neowiedii*' clade (clade C, Figs. 1-2). Contrary to the other two species groups presented above, there are no diagnostic morphological characters for this one. As stated before, it comprises two morphologically very distinct groups, the currently accepted species *M. ferdinandiana* and the *M. cogniauxiana – M. heterophylla – M. juergensii – M. neowiedii* group (or the '*M. neowiedii*' group). The data herein presented do not support these two groups as distinct clades. *M. ferdinandiana* is morphologically very distinct from other taxa in this clade, being easily characterized by its unifoliate and flat pseudobulbs, unique in the '*M. madida*' complex, and by its yellow flowers always shorter than the pseudobulbs. Another distinctive feature of this species is the presence of a single large metacentric chromosome pair with duplicated proximal $DAPI^+$ bands (Fig. 3a). Species from the '*M. neowiedii*' group can be distinguished from *M. ferdinandiana* by their bifoliate, cylindrical pseudobulbs and generally reddish to dark purple flowers, always longer than the adjacent pseudobulbs. Contrary to *M. ferdinandiana*, species delimitation within the '*M. neowiedii*' group is extremely unclear and vague, since diagnostic characters have shown, once more, to vary continuously among taxa. Such intricate morphological variation is well illustrated by the fact that one is able to observe, in the same locality, such as Campos de Jordão (São Paulo State, Brazil), individuals with flat, lanceolate leaves and reddish, smaller flowers blooming together with needle–like leaved specimens with larger, dark purple flowers, having intermediate morphotypes growing between them. Clearly the molecular markers used in this study did not present enough variation to distinguish between *M. ferdinandiana* and the '*M. neowiedii*' group. Considering our present lack of knowledge on phylogenetic patterns within this group, we suggest a conservative approach for species delimitation for the '*M. ferdinandiana – M. neowiedii*' clade based on morphological characters. Even though our results do not support these entities as monophyletic, morphological characters can be used, at least in a first instance, to distinguish *M. ferdinandiana* and a broad '*M. neowiedii*', as described above.

Cytogenetic data

Although the probable chromosome base number for the genus *Maxillaria* is x = 20 (Felix and Guerra, 2000), the optimization of chromosome numbers in the '*Maxillaria madida*' complex remains uncertain considering the available phylogenetic data at hand. Karyological studies considering Brazilian Maxillariinae indicate that species closely related to the '*Maxillaria madida*' complex have chromosome numbers of 2n = 38, 40 (J. S. Cabral, unpubl. res.) but the optimization of this character awaits further clarification of phylogenetic relationships concerning deeper nodes within the phylogeny of this subtribe (Whitten *et al*., unpubl. res.). We currently do not know the sister group of the '*Maxillaria madida*' complex and plastid and nuclear topologies indicate conflicting patterns of diversification within this group.

Cytogenetic data gathered for the '*Maxillaria madida*' complex show two general patterns. The species *M. ferdinandiana, M. heterophylla, M. pachyphylla* and *M. pumila* are characterized by 2n = 36, few CMA^+ bands and occurrence of submeta- and metacentric chromosomes, while *M. acicularis, M. madida* and *M. mosenii* have karyotypes with 2n = 38, more CMA^+ bands and apparent lack of submeta- and metacentric chromosomes. *M. uncata* might be included in the former group for sharing the same chromosome number, 2n= 36 (J. Cabral, unpubl. res.).

The occurrence of chromosome numbers of 2n = 36 broadens the record for the genus *Maxillaria* and suggests that disploidy – the increase or decrease of chromosome pairs – may be a common phenomenon in this group as in many other orchid groups (Brandham, 1999; Felix and Guerra, 2000). Disploidy has already been indicated as a mechanism of karyotype evolution in other groups of orchids, like in the section *Fimbriatae* of *Lycaste* (Ryan *et al*., 2000) and the genus *Paphiopedilum* (Cox *et al*., 1998). The probable mechanism involved in the differentiation of chromosome numbers in the '*Maxillaria madida*' complex is either centric fusion or centric fission (Robertsonian translocations), as supported by the banding pattern obtained for *M. ferdinandiana*. We observed in this species the presence of a single large metacentric chromosome pair with duplicated proximal $DAPI^+$ bands (Fig. 3a). The fact that acro- or telocentric chromosomes with proximal bands largely occurs in the species with 2n = 38 and that all the sampled species with 2n = 36 had at least one submeta– or metacentric chromosome pair with proximal $DAPI^+$ band, while none of the

species with 2n = 38 has shown this feature (Table 3, Fig. 3), also corroborates the occurrence of Robertsonian fusion/fission changes.

Considering the information above, the karyological data suggest that disploidy in the *'Maxillaria madida'* complex could be equally explained by two alternative evolutionary scenarios. First, one may consider that the ancestral of the *'Maxillaria madida'* complex already had a reduced chromosome base number of 2n = 36, which could be shared with its currently unknown sister group. In that case, the *'M. acicularis – M. madida'* clade would have increased the number of chromosomes (2n = 38) by centric fission, generating acrocentric or telocentric chromosomes with proximal $DAPI^+$ bands. The clade (*M. echiniphyta* + *M. pachyphylla* + '*M. ferdinandiana – M. neowiedii*' + '*M. pumila*') would have retained the ancestral state, presenting a base number of 2n = 36. Cytological data gathered for several Maxillariinae clades do not support this hypothesis since no other species outside the *'Maxillaria madida'* complex presents 2n = 36 (J. S. Cabral, unpubl. res.).

The other alternative scenario would be to have 2n = 38 as the pleisomorphic state for the *'M. acicularis – M. madida'* clade with the *'M. acicularis – M. madida'* clade having conserved the ancestral chromosome number. In this case, the common ancestral of the *'Maxillaria madida'* complex would have experienced further descendent disploidy to 2n = 36 due to centric fusion. Nevertheless, further cytogenetic studies considering additional samples, as well complementary phylogenetic data are necessary for an accurate inference of the evolution of cytological patterns in this group.

Conclusions

DNA sequence data were used to investigate species boundaries within the neotropical *'Maxillaria madida'* complex. Six currently accepted species were recovered by the phylogenetic analyses here presented (*M. echiniphyta*, *M. nardoides*, *M. pachyphylla*, *M. pacholskii*, *M. vernicosa* and *M. uncata*). Our results also support the recognition of the '*M. pumila*' clade as a single and polymorphic species based on diagnostic morphological characters as well by an overlapping geographic distribution of current species recognized within this clade. Two additional clades, the '*M. acicularis – M. madida*' clade and the '*M. ferdinandiana – M. neowiedii*' clade, demand further investigation since patterns of

diversification remained obscured within both groups. For now, we propose the recognition of a broadly defined *M. acicularis* for the '*M. acicularis* – *M. madida*' clade and two morphological complexes within the '*M. ferdinandiana* – *M. neowiedii*' clade (namely *M. ferdinandiana* and *M. neowiedii* s.l.) based on diagnostic morphological features described in the previous section. Complementary studies considering more populations as well as data from different molecular markers are already being developed in order to better understand diversification patterns within these clades. A pattern of heterochromatin distribution as well as karyotypic data certainly deserves greater attention as a valuable complementary source of information to understand evolutionary patterns within Maxillariinae orchids as well to assist the revision of species/generic boundaries within this subtribe. It is clear that speciation within Orchidaceae comprises a complex issue that must be explored in greater detail. The question of how such a vast range of morphological diversity was shaped and what is the role of ecology behind the formation of new species remains a fascinating subject to be further explored in this group of plants.

References

Atwood JT., Mora de Retana DE. 1999. Flora Costaricencis: Orchidaceae subtribes Maxillarinae and Oncidiinae. *Fieldiana, Botany n.s.* 40: 1–182.

Barbosa Rodrigues, J. (1996). Iconographie des orchidées du Brésil, vol. 1 & 2. Sprunger, S., Cribb, P. & Toscano de Brito, A. (eds.) Basle: Friedrich Reinhardt Verlag.

Bennett DE, Christenson EA. 1993.Plate 107: *Maxillaria nardoides* Kraenzl. In: Christenson EA., ed. *Icones Orchidacearum Peruvianum*. Privately published.

Bergsten J. 2005. A review of long–branch attraction. *Cladistics* 21: 163–193.

Blumenschein A. 1960. *Estudo sobre a evolução no subgênero* Cyrtolaelia *(Orchidaceae)*. Piracicaba: Escola Superior de Agricultura 'Luis de Queiroz', Universidade de São Paulo, Brazil.

Brandham P. 1999. Cytogenetics. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN, eds. *Genera Orchidacearum. Vol I.* New York: Oxford University Press, 67–80.

Buckley TR, Cordeiro M, Marshall DC, Simon C. 2006. Differentiating between hypotheses of lineage sorting and introgression in New Zealand alpine cicadas (Maoricicada Dugdale). *Systematic Biology* 55: 411-425.

Bull JJ, Huelsenbeck JP, Cunningham CW, Swofford DL, Waddell PJ. 1993. Partitioning and combining data in phylogenetic analysis. *Systematic Biology* 42: 384–397.

Butzin F, Senghas K. 1996. Subtribus: Maxillariinae. In: Brieger FG, Maatsch R, Senghas K, eds. *Rudolf Schlechter, Die Orchideen*. Berlin, Wien: Blackwell Wissenschafts–Verlag, 1727–1792.

Cabral JS, Felix LP, Guerra M. 2006. Heterochromatin diversity and its co–localization with 5S and 45S rDNA sites in chromosomes of four *Maxillaria* species (Orchidaceae). *Genetics and Molecular Biology*.29: 659-664.

Cabral JS, Felix LP, Guerra M. 2006. Heterochromatin diversity and its co–localization with 5S and 45S rDNA sites in chromosomes of

four *Maxillaria* species (Orchidaceae). *Genetics and Molecular Biology*,.29(4): 659-664.

Carpenter JM. 1994. Successive weighting, reliability and evidence. *Cladistics* 12: 177–6.

Christenson EA. 2003. Two Maxillarias, one old and one new. Orchid Review 111(1253): 287-289.

Cogniaux A. 1904. Orchidaceae III. Maxillarinae. In: von Martius CFP, Eichler AG, Urban I, eds. *Flora Brasiliensis. Vol. 3(6)*. Lipsiae: Frid. Fleischer, 1–78.

Cornelio MTMN, Figueiroa ARS, Santos KGB, Carvalho R, Soares WS, Guerra M. 2003. Chromosomal relationships among cultivars of *Citrus reticulata* Blanco, its hybrids and related species. *Plant Systematics and Evolution* 240: 149–161.

Cox AV, Abdelnour GJ, Bennett MD, Leitch IJ. 1998. Genome size and karyotype evolution in the slipper orchids (Cypripedioideae: Orchidaceae). *American Journal of Botany* 85(5): 681-687.

Cunningham CW. 1997. Is congruence between data partitions a reliable predictor of phylogenetic accuracy? *Systematic Biology* 46:464–478.

Dathe S, Dietrich H. 2006. Comparative molecular and morphological studies in selected *Maxillariinae* orchids. *Willdenowia* 36: 89–102.

Del Prete C, Mazzola P, Miceli P. 1991. Karyological differentiation and speciation in Mediterranean *Anacamptis* (Orchidaceae). *Plant Systematics and Evolution* 174: 115–123.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantaties of fresh leaf tissue. *Phytochemical Bulletin of the Botanical Society of America* 19: 11–15.

Dressler RL. 1993. *Phylogeny and Classification of the Orchid Family*. Portland, Oregon: Dioscorides Press.

Farris, J. S. 1969. A successive approximations approach to character weighting. *Systematic Zoology* 18: 374–385.

Farris JS, Källersjö M, Kluge AG, Bult C. 1994. Testing the significance of incongruence. *Cladistics* 10: 315–319.

Felix LP, Guerra M. 2000. Cytogenetics and cytotaxonomy of some Brazilian species of Cymbidioid orchids. *Genetics and Molecular Biology* 23: 957–978.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Fitch WM. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* 20: 406–416.

Goldman DH, Freudenstein JV, Kores PJ, Molvray M, Jarrell DC, Whitten WM, Cameron KM, Jansen RK, Chase MW. 2001. Phylogenetics of Arethuseae (Orchidaceae) based on plastid *matK* and *rbcL* sequences. *Systematic Botany* 26: 670-695.

Guerra M. 2000. Patterns of heterochromatin distribution in plant chromosomes. *Genetics and Molecular Biology* 23: 1029–1041.

Hillis DH, Huelsenbeck JP. 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83: 189–195.

Hoehne FC. 1947. Reajustamento de algumas espécies de Maxillarias do Brasil, com a criação de dois novos gêneros para elas. *Arquivos de Botânica do Estado de São Paulo* 2: 65–73.

Hoehne FC. 1952. Espécies e variedades novas de Orchidaceas do Brasil. *Arquivos de Botânica do Estado de São Paulo* 2: 121–136.

Hoehne FC. 1953. Orchidaceas. In: Hoehne FC. *Flora Brasilica. Vol 10 (12, 7)*. São Paulo, Secretaria da Agricultura, 223–341.

Holder MT, Anderason JA, Holloway AK. 2001. Difficulties in detecting hybridization. *Systematic Biology* 50: 978–982.

Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. *Trends in Ecology and Evolution* 11: 152–158.

Huelsenbeck JP, Hillis DM. 1993. Success of phylogenetic methods in the four-taxon case. *Systematic Biology* 42: 247-264.

Johnson LA, Soltis DE. MatK dna-sequences and phylogenetic reconstruction in Saxifragaceae s.str. *Systematic Botany* 19: 143-156.

Lee MSY. 2001. Uninformative characters and apparent conflict between molecules and morphology. *Molecular Biology and Evolution* 18: 676–680.

Lyons-Weiler J, Milinkovitch MC. 1997. A phylogenetic approach to the problem of differential lineage sorting. *Molecular Biology and Evolution* 14: 968-975.

Maddison WP, Knowles LL. 2006. Inferring phylogeny despite incomplete lineage sorting. *Systematic Biology* 55: 21–30.

Mansion G, Zeltner L, Bretagnolle F. 2005. Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae-Chironieae). *Taxon* 54: 931-950.

Martínez–Ortega MM, Delgado L, Albach DC, Elena–Rosselló JA, Rico E. 2004. Species boundaries and phylogeographic patterns in cryptic taxa inferred from AFLP Markers: *Veronica* subgen. *Pentasepalae* (Scrophulariaceae) in the Western Mediterranean *Systematic Botany* 29: 965–986.

Onishi E. 1974. Sobre o polimorfismo do grupo *Maxillaria madida* (Orchidaceae, Maxillarinae). PhD Thesis, Escola Superior de Agronomia Luis de Queiroz, Universidade de São Paulo, Brazil.

Pabst GFJ, Dungs F. 1977. *Orchidaceae Brasiliensis II*.Hildesheim: Brücke–Verlag Kurt Schmersow.

Parker LT, Koopowitz H. 1993. Chloroplast DNA and the determination of species status in the *Disa tripetaloides* complex (Orchidaceae), and its relationships to 3 species Racemosae, Section Disa. *Biochemical Systematics and Ecology* 21: 799–808.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

Reed RD, Sperling FAH. 1999. Interaction of process partitions in phylogenetic analysis: An example from the swallowtail butterfly genus *Papilio. Molecular Biology and Evolution* 16: 286–297.

Ryan A, Whitten WM, Johnson MAT, Chase MW. 2000. A phylogenetic assessment of *Lycaste* and *Anguloa* (Orchidaceae: Maxillarieae). *Lindleyana* 15: 33-45.

Schweizer D, Ambros PF.1994. Chromosome banding. In: Gosden JR, ed. *Methods in Molecular Biology. Vol 29.* Totowa: Humana Press, 97–112.

Stebbins GL. 1966. Chromosomal variation and evolution. *Science* 152: 1462–1469.

Stergianou KK. 1989. Habitat differentiation and chromosome evolution in *Pleione* (Orchidaceae). *Plant Systematics and Evolution* 166: 253–264.

Sun Y, Skinner DZ, Liang GH, Hulbert SH. 1994.Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribossomal DNA. *Theoretical and Applied Genetics* 89: 26–32.

Swofford DL 2000. *PAUP*: Phylogenetics analysis using parsimony (and other methods), version 4.0b10.* Sunderland, Massachusetts: Sinauer Associates.

Swofford DL, Olsen, GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular Systematics. 2nd Ed.* Sunderland, Massachussets: Sinauer, 407-514.

Taberlet PL, Geilly G, Bouvet J. 1991. Universal primers for amplification on three non–coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

Whitten WM, Williams NH, Chase MW. 2000. Subtribal and generic relationship of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. *American Journal of Botany* 87: 1842–1856.

Wolfe AD, Randle CP. 2004. Recombination, heteroplasmy, haplotype polymorphism, and paralogy in plastid genes: implications for plant molecular systematics. *Systematic Botany* 29: 1011–1020.

Acknowledgements

We are much indebted to the institutions which provided specimens for study as well for all the help of their staff: Instituto de Botânica de São Paulo (E. Catharino, F. de Barros, F. Pinheiro, R. Peres and H. Michelan), Escola Superior de Agricultura "Luiz de Queiroz" (E. Ann–Veasey, G. Oliveira, J. Pontes) and Ecuagenera Ltd. (Portilla family and Ecuagenera staff). We sincerely thank A. P. de Moraes, J. Aranha, L. Kollmann, R. Custódio, R. Farias–Singer, R. Gonçalves for all the lab assistance and/or help on fieldwork and A. dos Santos, A.O. Simões, G. Machado, J. Joseph, V. Bittrich for valuable discussions on phylogenetic analyses. Plants were collected under the permit No. 016–IC–FLO–DBAPMA from the Ministerio del Ambiente of Ecuador. This work was supported by funding form Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to S. Koehler (02/02161–7), R.B. Singer (01/08958–1), and M.C.E. Amaral (02/02162–3); by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to J.S. Cabral and M. Guerra, by a Prance Fellowship in Neotropical Botany from the Royal Botanical Gardens Kew to S. Koehler and the U.S. National Science Foundation grant No. DEB–234064 to N.H. Williams and W.M. Whitten for the project "Systematics of Maxillariinae (Orchidaceae): generic delimitation, pollinator rewards, and pollination"

2

DELIMITAÇÃO DE ESPÉCIES NO CLADO '*MAXILLARIA ACICULARIS* – *MAXILLARIA MADIDA*' BASEADA EM MARCADORES AFLP

Species limits in the *"Maxillaria acicularis – M. madida"* clade (Orchidaceae) based on Amplified Fragment Length Polymorphism (AFLP) markers

Samantha Koehler[1,2*], Michael F. Fay[3], Volker Bittrich[2], Maria do Carmo E. Amaral[2]

[1] Pós–graduação em Biologia Vegetal and

[2] Departamento de Botânica, IB, Universidade Estadual de Campinas, Campinas, SP, 13083–970, Brazil

[3] Jodrell Laboratory, Royal Botanical Gardens, Kew, Richmond, Surrey, TW93DS, UK

* For correspondence. E–mail samantha.koehler@gmail.com

Key words: species delimitation, species concepts, Maxillariinae, neighbor-joining, principal coordinates analysis, campos rupestres, mata atlântica

Introduction

The nature and origin of species are central issues in evolutionary biology. More than the consequence of taxonomic categorization of living things, species represent the taxonomic units with the most tangible connection to populations and individuals, which are the fundamental units of biodiversity. Studies on species diversification have increased considerably in the past decade as questions regarding close kinship grade into those of population structure and infraspecific phylogeny and subsequently into speciation and associated taxonomic judgments (Avise 2004). But although species is a fundamental unit of all biological studies, there is little consensus on the appropriate concept of species (Mayr 1982, Howard & Berlocher 1998, Coyne & Orr 2004, Mallet 2006).

The first question we must ask when trying to define species is what exactly species are. Underlying the debate for an universal concept is the reality of species. Most biologists certainly act as if species are real: systematists identify specimens as species and use them to reconstruct the history of life based on character states restricted to species; population geneticists measure genetic diversity within species and ecologists calculate species diversity (Coyne & Orr 2004). Therefore, according to the reasons above, species do comprise discrete units of nature. On the other hand, species have also been considered as subjective divisions of nature made for human convenience only (Darwin 1859, Sokal & Crovello 1970, Mallet 1995a, 1997). According to them, populations in nature exhibit a continuous range of variation and we can only arbitrary define species, as humans have a propensity to classify things and a need for designating operational units. In an attempt to answer this question, Coyne & Orr (2004) gathered data that demonstrates that "folk" and "scientific" species are generally concordant and that sophisticated statistical analyses may be applied to confirm the existence of distinct clusters of organisms living in sympatry. These findings corroborate the existence of species as real entities, especially for most sexually reproducing organisms (Coyne & Orr 2004).

The next question to be considered is: given that species are real, how do we define them? Until the 1980's, two species concepts dominated biological studies: the typological or morphological concept (Mayr 1959) and the Biological Species Concept (BSC, Mayr 1942). The typological concept dominated the nineteenth century and up today it is still

largely applied, since it is based on the general morphological similarity among organisms. It remains the most useful concept for taxonomists working in groups with many undescribed species because it is efficient in the majority of cases (Berlocher 1998). While the typological concept is now generally disregarded due to its lack of theoretical conceptualization, the search for a definable, ideal reality of a species - despite imperfect manifestations of that ideal in nature - may be the legacy of typological thinking in biology (Carvalho & Hauser 2000).

The most well known species concept is probably the Biological Species Concept (BSC). First suggested by Poulton (1908) and later formally recognized as so by Mayr (1942), it defines species as a group of populations that are reproductively isolated from others. Despite its popularity due to the testability in sexual species in a local context, critics of the BSC have pointed out several examples of ambiguities of species status and the existence of groups to which the concept cannot be applied (Mallet 1995a and references). One of the major problems with the BSC is the occurrence of geographically isolated and morphologically differentiated populations. Such populations are difficult to categorize using the BSC because their ability to coexist in sympatry is practically untestable. Successful crossing by these forms in captivity/cultivation, when viable, does not prove conspecificity – orchids frequently hybridize under artificial conditions (van der Pijl & Dodson 1966), even between genera not closely related. Most important, some authors claim that the BSC runs into theoretical difficulties when used in tests of hypotheses of speciation - since speciation involve a reduction in the ability to interbreed, species cannot be defined by interbreeding without confusing cause and effect (Mallet 1995a).

A second group of concepts considers genetic/phenotypic cohesion. The Recognition Concept (Paterson, 1985), in which species are defined as "specific-mate recognition systems" also uses reproductive traits to define entities denominated species and, therefore, comprises a sub-concept within the BSC. Sokal & Crovello (1970) proposed that clusters of individuals based on multivariate analysis could be recognized as phenetic species. Following basically the same idea, Mallet (1995a) suggested species recognition should be based on principals not only of phenotypic clustering, but also on genotypic differences, mainly in zones of geographical overlap, with few or no intermediates in

contact between clusters (Genotypic Cluster Species Concept, GCSC). The main difference between the BSC and concepts based on clustering such as the GCSC is that the former define species as groups of inbreeding populations, while the later uses phenotypic/genetic data to recognize such clusters, accepting, therefore, a certain degree of gene flow. The BSC fails to deal with gene flow, especially when gene exchange occurs between sympatric morphological distinct taxa, such as observed for many birds and plants (Coyne & Orr 2004). The main problem of the GCSC is probably the level of clustering appropriate for species recognition – because of the hierarchical nature of evolution, genotypic clusters more or less well defined occur at many levels, such as polymorphic groups within populations (Sites & Crandall 1997, Coyne & Orr 2004). To get around this problem one must often include a reproductive criterion, which defeats the GCSC's goals of avoiding it. As an alternative, Templeton (1989) proposed the Cohesion Species Concept (CSC) in which species are defined by cohesion mechanisms (reproductive isolation, recognition mechanisms, ecological niche) and genealogical distinctness. The CSC gives a mechanistic support to the GCSC by considering all factors that preserve morphological and genetic clusters, that is, it incorporates explanations for why individuals within clusters remain genetically/phenotypically similar (Coyne & Orr 2004). In many cases involving sympatric sexual taxa, both the BSC and the CSC recognize the same clusters (reproductive isolating barriers are recognized as cohesion mechanisms by the CSC) (Coyne & Orr 2004). Also, when forced crosses show that allopatric populations have incomplete isolation, there is no way to diagnose these populations under the CSC, as is the case for the BSC (Coyne & Orr 2004). In addition, both concepts have problems to deal with groups that despite some degree of gene exchange remain distinct (Mallet 1995b, Sites & Crandall 1997, Coyne & Orr 2004).

A third group of species concepts is based on evolutionary cohesion. The Evolutionary Concept (EvSC, Simpson 1951) appeared as an early attempt to define species based on history, which were defined as "a lineage evolving separately and with its own unitary evolutionary role and tendencies". The EvSC differs from the BSC by including no explicit mention of genetic interchange or reproductive isolation. Nevertheless, the arguments in favour of the EvSC show that, in most cases, it is equivalent to the BSC, at least for diagnosing species in sympatry: "separate evolutionary

lineages must be reproductively isolated from one another to the extent that this is required for maintaining their separate identities (Wiley 1978, Coyne & Orr 2004). Similarly, the Ecological Concept (EcSC, Van Valen 1976) was proposed considering "species as a lineage which occupies an adaptive zone minimally different from that of any other lineage". The EcSC resembles the EvSC except that independently evolving lineages are characterized as occupying "minimally different adaptative zones" (Van Valen 1976, Coyne & Orr 2004).

The establishment of the phylogenetic systematics (Hennig 1950) fuelled the appearance of new species concepts based on evolutionary history due to the application of the principles of cladistics at species rank. Hennig's cladistic concept considered a species as "an unbranched lineage in an organismal phylogeny" (Hennig 1966). The idea behind Hennig´s concept is that "any [species] concept potentially useful in phylogenetic systematics must precisely specify limits of species in time" (Meier & Willmann 2000). Perhaps the main criticism of this idea is that it could, if applied in taxonomy, cause great nomenclatural instability (Mallet 1995a, 2006). Monophyly can exist at very high or very low levels of the phylogeny, so the precise level at which species taxa exist becomes unclear. Another problem with a monophyly concept at species level is that a single, true phylogeny of a species may rarely exist: an organismal phylogeny is in fact an abstraction of the actual genetic history, consisting of multiple gene genealogies, some of which may undergo genetic exchange with other species. There is now good evidence that occasional horizontal gene transfer and hybridization may selectively transfer genetic material between unrelated forms (Coyne & Orr 2004). Furthermore, there are multiple gene lineages within any population, so that, if such a population were to become geographically or genetically split into two distinct forms, it would be some time before each branch became fixed for different, reciprocally monophyletic gene lineages at any single gene. The idea of monophyly for whole genomes then becomes hard to define, especially near the species boundary (Mallet 2006).

Although Phylogenetic Species Concepts (PSC) converge with the BSC in recognizing most sympatric species (diagnosed as monophyletic groups defined by synapomorphies), there are discordances. If one uses interbreeding to define species originated through peripatric and parallel speciation, non-monophyletic groups will be recognized. Under the

PSC, these populations are lumped into a single species because of the absence of reciprocal monophyly, while the BSC recognizes two species based on reproductive isolation (Meier & Willmann 2000, Coyne & Orr 2004). On the other hand, the PSC allows a great deal of splitting – even if divergence is undetectable by phenotypic diagnostic characters splitting is consented (Sites & Crandall 1997).

A more practical phylogenetic concept, the Diagnostic Concept, was proposed by Cracraft (1989), which defined species as "an irreducible cluster of organisms, diagnosable distinct from other such clusters, and within which there is a parental pattern of ancestry and descent". Although this concept reduces the amount of splitting, it allows the recognition of non-monophyletic species (but see McKitrick & Zink 1988 for more restrictive considerations). When considering phylogenetic species definitions one must bear in mind that the underlying process of gene genealogical divergence in lineage splitting may be (and usually is) much more complex than depicted because of differential inheritance of genes (ancestral polymorphism) (Avise & Ball 1990).

Avise & Ball (1990) proposed another version of a phylogenetic concept denominated the Genealogical Species Concept (GSC) as a way to diagnose phylogenetic status of populations using independent phylogenies (based on unlinked and non-epistatic loci) and avoid the problem of ancestral polymorphism. Like the previous PSC, the GSC recognizes species as exclusive groups whose members are more closely related to each other than to individuals of other species – the difference being is that the GSC explicitly defines monophyly of taxa as the monophyly of genes carried by its members (Avise & Ball 1990). The rationale behind the GSC is that concordance among independent gene genealogies is likely to arise when populations have been reproductively separated from one another by intrinsic or extrinsic barriers for a long period of time (Avise & Ball 1990). A drawback of the GSC is the difficulty of obtaining complete monophyly of all genes sampled – genealogical species have been diagnosed on the basis of a single haplotype or allozyme locus (Young & Crother 2001, Leaché & Reeder 2002). For that reason it has been suggested that only a proportion of the sampled loci must be monophyletic to allow GS status. More relaxed criteria have been proposed (Shaw 2001), varying from 50% to 95% of monophyletic loci, but the number of genes/loci necessary to diagnose a species remains debatable. It is important to notice that the time to genealogical speciation decreases as the

GS criterion gets more relaxed. While a more relaxed GS criterion seems most reasonable, it allows a possible omission of species that may have evolved recent intrinsic reproductive barriers and, therefore, lineage separation and concordance will not be evident for most markers (Avise & Ball 1990, Sites & Crandall 1997). For this reason, subjective judgements may be required for intermediate levels of congruence/divergence such as differences in additional attributes (e.g. morphology and behaviour) (Sides & Crandall 1997).

Despite the fact the number of species in a certain group may depend on the species concept used, the diversity of concepts available have hardly affected taxonomic practice, which appears to be concerned primarily with the identification of morphological and genotypic clusters (Mallet 1995a, Carvalho & Hauser 1999). This is especially true when considering the description of new species, in which morphological characters are still the main source of information (typological concept), without major concern about species concepts. Nevertheless, it is clear that for some groups of organisms the morphological data available appear to be inadequate for species delimitation due to the mosaic distribution of diagnostic characters, both within and between currently defined taxa. Consequently, the use of molecular characters, potentially more numerous and informative, offers a possible means of resolution of the problem of species delimitation.

Study system

The '*Maxillaria madida*' complex presently includes ten species (S. Koehler et al., unpubl. data), distributed mainly throughout the southern and south-eastern regions of Brazil, with a few species occurring from northern South America to southern Mexico. A phylogenetic study of this group based on DNA sequence data from multiple regions, including nuclear and plastid genomes (S. Koehler, unpubl. data), indicated that two presently accepted species, *M. acicularis* Herb. ex Lindl. and *M. madida* Lindl., including *M. mosenii* Kraenzl. (following Pabst & Dungs 1977), constitute a monophyletic group restricted to the south and south-eastern regions of Brazil. The sequence data, however, were unable to distinguish between these two taxa (Fig. 1). Although morphologically very similar, *M. acicularis* and *M. madida* have been traditionally differentiated by the shape of the leaves and flower size (Hoehne 1953, Pabst & Dungs 1977).

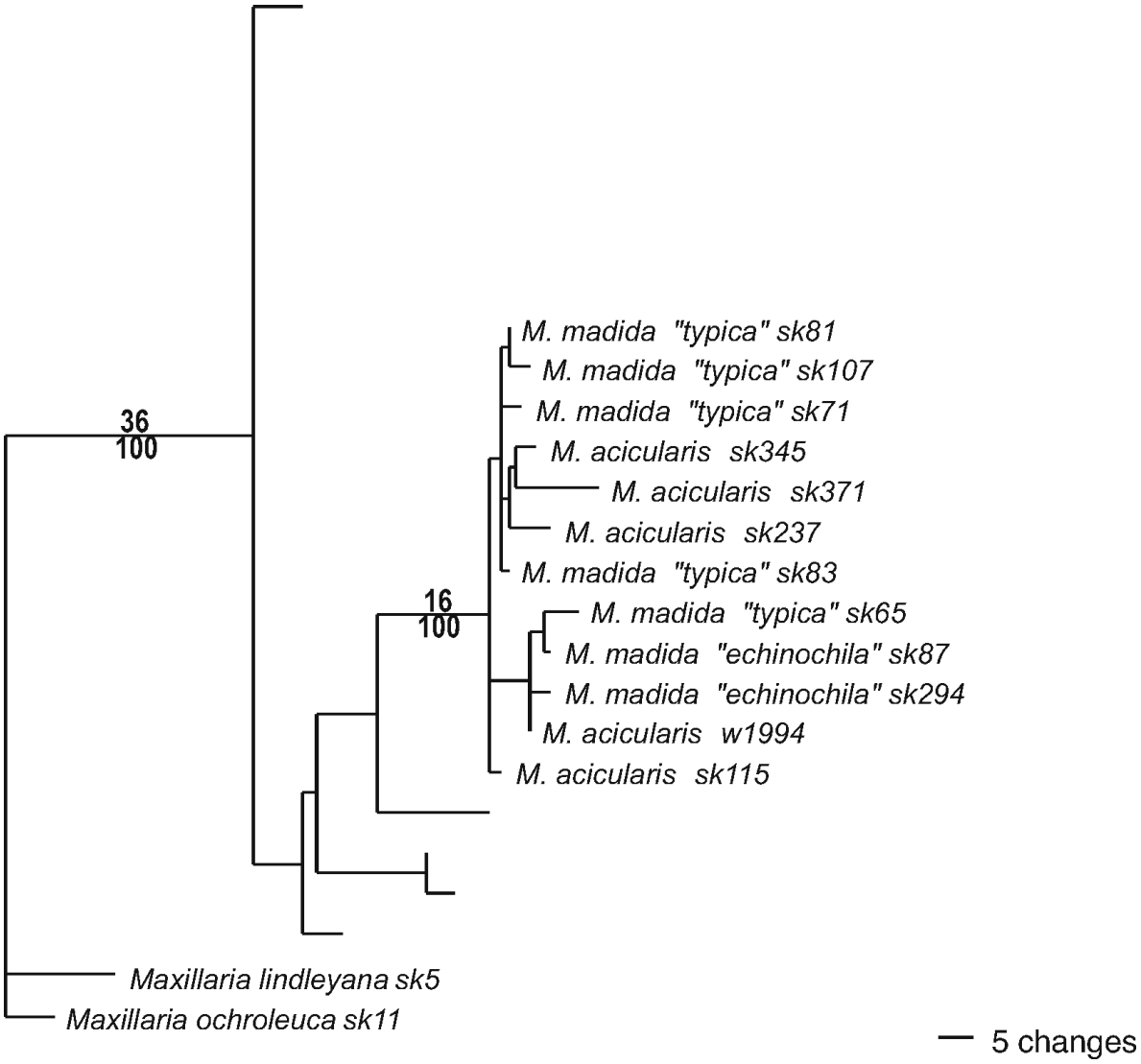


Fig. 1. Partial phylogram of the *'Maxillaria madida'* complex based on DNA sequence data from nuclear and plastid genomes (Koehler et al., unpubl. data). Only samples belonging to the *M. acicularis - M. madida* clade are shown. Branch lengths/bootstrap values are indicated for the *'Maxillaria madida'* complex and *M. acicularis - M. madida* clade.

Plants belonging to *M. madida* have been traditionally recognized as more robust, with larger pseudobulbs and flowers, and with two leaves (rarely one) varying from linear-lanceolate to cylindrical, whereas *M. acicularis* corresponds to more delicate plants, with smaller pseudobulbs, bearing only two needle-like leaves and smaller flowers (Fig. 2). Since leaf morphology is extremely variable among populations of *M. madida*, different ideas of species delimitation have been proposed based on different aspects of morphological variation (Cogniaux 1904, Hoehne 1953, Onishi 1974, Pabst & Dungs 1977, Butzin & Senghas 1996; Table 1). Out of all the names and taxonomical varieties available, one is able to generally recognize four morphotypes according to vegetative characters, as indicated in Table 1 and Fig. 2. Interestingly, morphological variation in this group seems to be restricted ecologically and/or geographically as already pointed out by previous authors (Hoehne 1953, Onishi 1974). The morphotype "echinochila" recognized by Hoehne as *M. mosenii* var. *echinochila* (Kraenzl.) Hoehne is restricted to the rocky outcrop formations of the Serra do Espinhaço and Mantiqueira mountain ranges, in the state of Minas Gerais, south-eastern Brazil (Hoehne 1953) (Figs. 2 and 3). Hoehne (1947) also proposed the new combination *M. mosenii* var. *typica* (nom. inval., ICBN §24.3) as restricted mainly to the seasonally dry forests and Atlantic rainforests of south-eastern Brazil.

Posteriorly Onishi (1974), in an unpublished study based on morphometric analysis of the species *M. madida* and *M. mosenii*, recognized *M. madida* subsp. *unifolia* (nom. nud.) as another morphologically distinct group restricted to north of Espírito Santo and south of Bahia (Table 1; Figs. 2 and 3). Despite the fact that *M. acicularis* and *M. madida* are morphologically very similar, with diagnostic characters varying continuously among species and infraspecific categories with ecological/geographic overlapping (Figs. 2 and 3), species boundaries between them have never been questioned (Hoehne 1953, Onishi 1974).

Table 1. Former classifications proposed for the *Maxillaria acicularis* – *M. madida* clade, with diagnostic characters indicated for each morphotype.

MORPHOTYPE (as here recognized)	**DIAGNOSTIC CHARACTERS**	**COGNIAUX (1904, 1906)**	**HOEHNE (1953)**	**ONISHI (1974)**	**PABST & DUNGS (1977)**	**BUTZIN & SENGHAS (1996)**
"acicularis"	more delicate smaller plants, two needle-like leaves, smaller flowers	*M. acicularis*	*M. acicularis*	*	*M. acicularis*	*M. acicularis*
"echinochila"	robust plants, larger flowers, two extremely convolute, thick leaves	*M. madida*	*M. mosenii* var. *echinochila*	*M. madida* subsp. *acicutifolia*	*M. madida*	*M. mosenii*
"monophylla"	robust plants, larger flowers, one long cylindrical leaf	*M. madida* var. *monophylla*	**	*M. madida* subsp. *unifolia*	**	**
"typica"	robust plants, larger flowers, two flat, coriaceous, lanceolate leaves	*M. madida*	*M. mosenii* var. *typica*	*M. madida* subsp. *madida*	*M. madida*	*M. madida*

(*) Taxa not considered in the study developed by Onishi (1974)

(**) Taxa unknown to author. Onishi´s study was concluded in 1974 but never published.

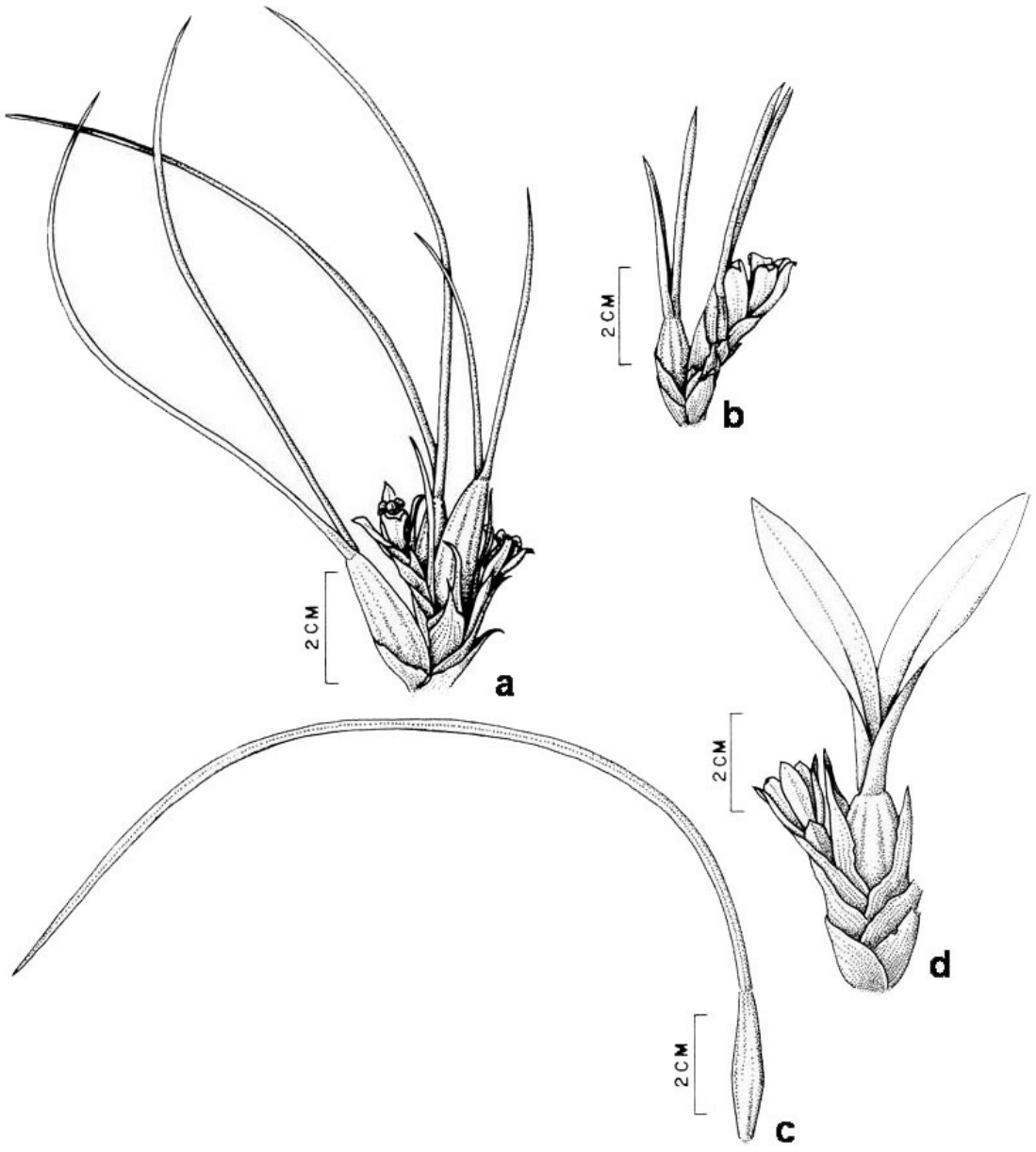


Fig. 2. Vegetative morphs found within the *Maxillaria acicularis-M. madida* clade. (a) "acicularis", (b) "echinochila", (c) "monophylla", (d) "typica".

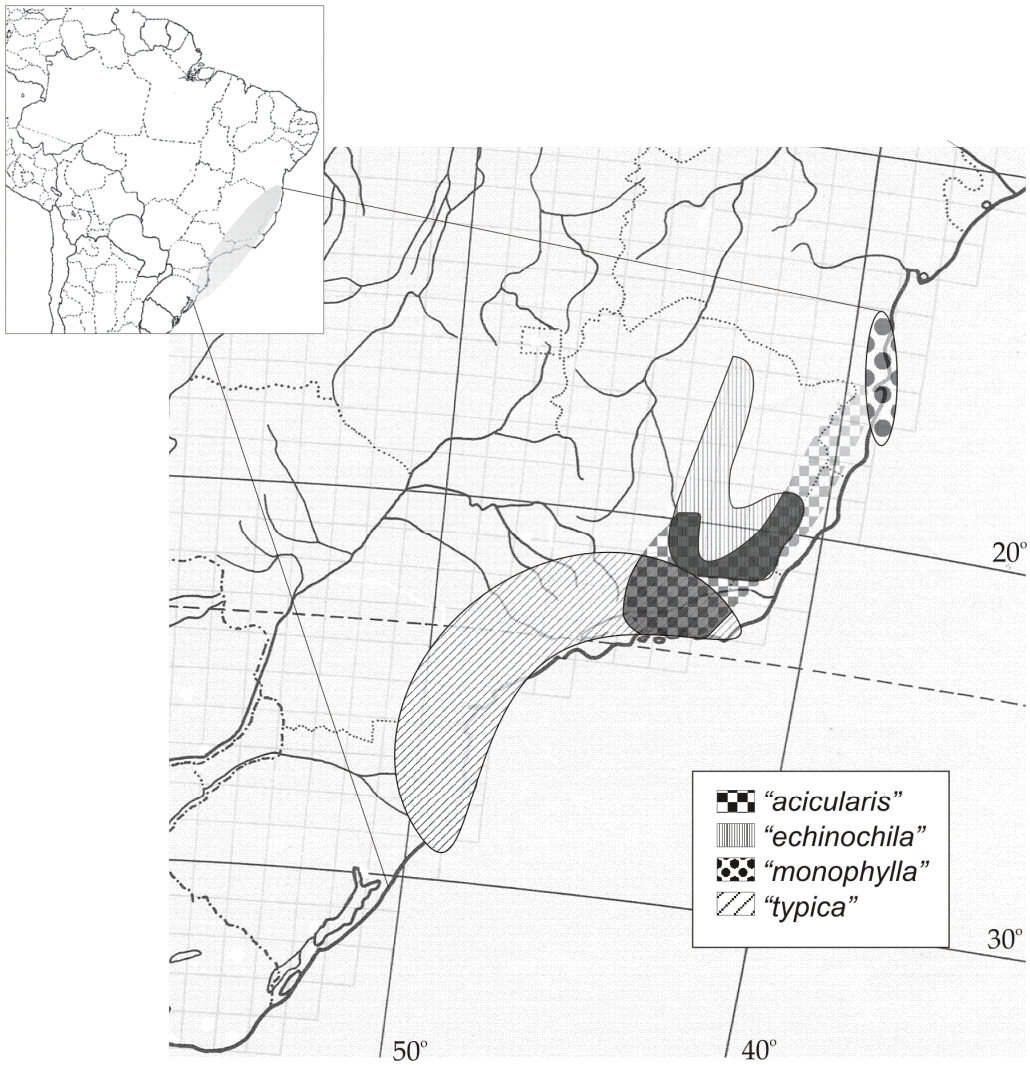


Fig. 3. Map indicating the distribution of morphotypes. Shaded areas correspond to overlapping distributions.

Molecular markers

Developments in molecular methods have made significant contributions to the fields of taxonomy and systematics of flowering plants, including the family Orchidaceae. Besides assisting in the revaluation of the taxonomic status of complex groups (e.g. Squirrell et al. 2002, Hedrén 2003, Goldman et al. 2004), molecular analysis has also prompted reinterpretation of the evolution of phenotypic characters, as well as the estimation of age and rate of diversification of lineages (e.g. Barraclough & Savolainen 2001, Gustafsson & Lönn 2003). In addition, molecular data may guide the analysis of distribution patterns in a historical and ecological perspective as well as the understanding of mechanisms of speciation (e.g. Hedrén et al. 2001, Cozzolino et al. 2003a, Cozzolino et al. 2003b, Gustafsson & Lönn 2003, Hedrén et al. 2001, Wallace 2003a). Molecular data have also been employed in the investigation of species status in orchids (e.g. Squirrell et al. 2002, Wallace 2003a, Wallace 2003b). Indeed, the increasing availability of molecular techniques provides a generally applicable and objective approach for the comparative analysis of population and species identity.

Amplified fragment length polymorphisms (AFLP) were primarily applied to genome mapping (Vos et al. 1995), but the high polymorphism revealed by AFLPs almost immediately interested molecular systematists and population geneticists. Within the family Orchidaceae, some studies have used AFLPs to assess genetic diversity and population structure (e.g. Forrest et al. 2004, Smith et al. 2004), while others had more specific goals, such as investigating the origin of hybrid taxa (e.g. Hedrén et al. 2001, Goldman et al. 2004). Recently, AFLP markers have also been employed to resolve phylogenetic relationships at low taxonomic levels, especially when DNA sequence data fail to provide enough variability (e.g. Hedrén et al. 2001, Després et al. 2003, Goldman et al. 2004). The major advantage of the AFLP technique is the large number of polymorphisms that the method generates, allowing a high number of different loci to be simultaneously analyzed per experiment. Also, the complete genome is densely sampled with no prior sequence information required for the development of this technique (Mace et al. 1999; Mueller & Wolfenbarger 1999). Additionally, silica-gel dried material can be used for the analyses, since the method is DNA-based, enabling the analysis of species that would be otherwise difficult to sample (Harris & Robinson 1994, Russell et al. 1999).

Nevertheless, there are limitations to the use of AFLP in phylogeny, with homology being perhaps its greatest limitation (Hillis 1994). Even though, numerous studies have shown that the analysis of AFLP markers yields relationships that are congruent with those suggested by phylogenetic studies based on morphological characters or on nuclear/chloroplast sequence data (e.g. Kardolus et al. 1998, Mace et al. 1999, Goldman et al. 2004). At the lower taxonomic levels to which these markers are usually applied, homoplasy of AFLP bands apparently is not frequent enough to obscure evolutionary relationships (El-Rabey et al. 2002).

It is inappropriate to assume that molecular markers can provide the final answer to species identification. Ideally, also for practical reasons, phenotypic characters, as well as geographic and/or ecological data, should be considered in the taxonomic delineation of species complexes. The advantage of a combined molecular and morphological approach in this case is that one can assess genetic variability as well as evidence of gene flow – which is crucial for the conservation of biodiversity – and gather, hopefully, enough phenotypic data for the identification of species and/or infraspecific taxa by taxonomists.

In this study we propose to investigate the taxonomic status of *M. acicularis* and *M. madida* (including *M. mosenii*) as distinct species and suggest the recognition of taxonomic units according to the patterns of genetic and morphological variation. In order to do that we undertook a comparative study based on AFLP markers of different populations throughout the geographical and ecological range of *M. acicularis* and *M. madida*. Species limits will be tested under the assumptions of the Genetic/phenotypic Cohesion Species Concept (GCSC, Mallet 1995a), in which a species is defined as "a morphologically or genetically distinguishable group of individuals that has few or no intermediates when in contact with other such clusters" (Mallet 1995a).

There are three main reasons why debates concerning species concepts persist (Coyne & Orr 2004): (1) there is no concept free of ambiguities; (2) every strict concept fails in some situations; (3) biologists want species concepts to be useful for some purpose, but differ in what that purpose should be. In this study, we followed Coyne & Orr (2004) in identifying a species concept that suites the nature of our "species problem". For that reason we justify the choice of the GCSC as follows.

First, we need a concept that help us to classify entities within the '*M. acicularis* - *M. madida*' clade - in which morphological characters are known to have a mosaic distribution among taxa - in a systematic, efficient manner, allowing the identification of groups in the field and herbarium specimens. This is perhaps the major advantage of using the GCSC concept, since it advocates the use of patterns of discrete genetic differences to reveal clusters (Mallet 1995a). Secondly, given that AFLP data should be applied in phylogenetic studies with caution and preferably in combination with other types of markers (Swofford et al. 1996), it is important to consider a concept not based on exclusively phylogenetic reconstructions. Again the GCSC suites our problem, since it does not require knowledge on the evolutionary history of the group in order for species to be defined (Mallet 1995a). A last important consideration is that the GCSC is well suitable for organisms in which reproductive isolation is difficult to demonstrate through artificial experiments, such are orchids. Also, when there are geographical or ecological separation of populations, field observations are also of little help to demonstrate reproductive isolation. Unlike the BSC and other concepts, the GCSC defines species solely by the features used to recognize them (Mallet 1995a, Coyne & Orr 2004).

Materials and methods

Sampling was performed including all morphological variants of *M. acicularis* and *M. madida*, denominated here as morphotypes. DNA from fresh plant tissues (leaves and flowers) of 56 cultivated specimens (Table 2) was extracted according to Doyle and Doyle (1987) and scaled down to 1 ml extraction volumes following the protocol described in Whitten et al. (2000). All DNA samples were posteriorly purified using QIAquick columns (Qiagen Inc.) to eliminate PCR inhibitors known to occur in this group of orchids (M. Whitten, pers. comm.).

Table 2. Samples of morphotypes within the "*Maxillaria acicularis - M. madida*" clade. State abreviations are as follows: BA, Bahia; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná. All vouchers were deposited at ESA.

morphotype	habitat type	locality (# ind. sampled)	Abreviations for populations	voucher
"acicularis"	humid North	Floresta Azul, BA (2)	FLAZ	*Koehler 125*
	humid South	Nova Friburgo, RJ (3)	FRIB	*Koehler 146*
"echinochila"	dry	Caeté, MG (3)	CAETE	*Koehler 167*
	dry	Presidente Juscelino, MG (3)	JUCE	*Koehler 193*
	dry	Ouro Preto, MG (3)	OUPR	*Koehler 158*
	dry	Santana do Riacho, Serra do Cipó, MG (8)	CIPO	*Koehler 6*
"acicularis" - "echinochila"	dry	Ervália, MG (3)	ERVA	*Koehler 116*
	humid South	São Fidélis, Poço Parado, RJ (3)	SFID	*Koehler 108*
"typica"	humid South	Angra dos Reis, RJ (3)	ANGRA	*Koehler 100*
	humid South	Capão Bonito, SP (3)	CAPAO	*Koehler 71*
	humid South	Curitiba, PR (2)	CURI	*Koehler 95*
	humid South	Guapiara, SP (3)	GUAP	*Koehler 90*
	humid South	Itatiaia, RJ (2)	ITAT	*Koehler 61*
	humid South	Mogi das Cruzes – Bertioga, SP (3)	MOBER	*Koehler 153*
	humid South	Santo André, Paranapiacaba, SP (2)	PNPC	*Koehler 168*
"monophylla"	humid North	Santa Luzia, BA (2)	STLUZ	*Koehler 133*
	humid North	Santa Maria do Salto, MG (5)	SALTO	*Koehler 29*
	humid North	Una, BA (3)	UNA	*Koehler 128*

An automated AFLP procedure using fluorescent dyes was carried out as described in the AFLP Plant Mapping Protocol (1996, Applied Biosystems Inc.). First, digestion of genomic DNA with the *EcoRI* and *MseI* restriction enzymes was performed, together with the ligation of double-stranded DNA sequences (adaptors) to the ends of the restriction fragments. Then, two rounds of PCR amplification followed. The first used standard preselective primers that recognize the adaptors plus a single nucleotide in the original restriction fragment. For the second PCR reaction, 16 different primer combinations were tested and three were chosen for this study (AGG-CAA, ACT-CAC, AAC-CAC). Fluorescently labelled fragments from the selective amplification were visualized using an ABI 3100 automated sequencer, according to the manufacturer's protocols (Applied Biosystems Inc.). Despite the previous DNA purification procedure, we had to modify the original protocol due to the presence of inhibitors, as previously observed for other species within the genus *Maxillaria* (M. Whitten, pers. comm.). In order to obtain successful results, we slightly modified the procedure of Indsto et al. (2004), in which very small quantities of DNA were used in the restriction-ligation reaction (10 - 100 ng) with no dilution of restriction-ligation products previous to pre-selective amplifications.

Data analysis was carried out using GeneScan 3.1 and the bands were evaluated by size and scored in Genotyper 2.0 (Applied Biosystems Inc.) in two steps. First, a pilot analysis was performed based on 18 species and seven populations to check if the selected primer combinations had enough variation both within and among populations. A second and final analysis included all 56 individuals and 18 populations. Binary matrices listing presence/absence of each band for all samples was created from the AFLP data and analysed by the neighbor-joining (NJ) distance algorithm (Sneath & Sokal 1973), the maximum parsimony (MP) criterion (Fitch 1971), and the principal coordinates ordination method (PcoA, Gower 1966). The NJ analysis was carried out in PAUPb10* (Swofford 2000) using Nei & Li (Nei & Li, 1979) distance coefficient, which measures the probability that a band being amplified in one sample is also amplified in another sample (Robinson & Harris 1999). This coefficient is also an estimate of the proportion of the number of bands shared by two samples because they were inherited from a common ancestor (Harris 1999). The search strategy for the MP analysis, also performed in PAUPb10*, was based on 1000 addition sequence replicates by stepwise addition holding 10 trees/replicate, TBR

branch swapping on best trees, MULTREES on, excluding uninformative characters and with ACCTRAN optimization. Trees were rooted using the midrooting procedure of PAUP*. Bootstrapped pseudo-replicates (1000) were generated to check for confidence limits on the obtained results of both NJ and MP analyses. The categories of bootstrap support (BS) considered in this study were: unsupported (<50%); weak (50–74%); moderate (75–84%); strong (85–100%). PcoA analyses were performed in R4.0d0 (Casgrain & Legendre 1998), using Jaccard's coefficient (Jaccard 1908), which excludes similarity due to shared absences. The table of eigenvalues produced by the R program was exported to Microsoft Excel to produce XY scatter plots.

Results

Scored fragments ranged from 84 to 461 bp. In the pilot analysis (18 samples, 7 populations) the number of interpretable bands obtained for the three primer combinations was 338, of which 228 (67.4%) were polymorphic. For the final analysis (56 samples, 18 populations) we excluded results obtained for the primer combination AAC-CAC due to the poor quality of the data. The number of interpretable bands obtained for the two primer combinations combined was 220, of which 190 (86.4%) were polymorphic.

Pilot analysis

In the preliminary analysis, the NJ phenogram does not depict the populations of *M. acicularis* and *M. madida* as forming two distinct clusters (Fig. 4). Instead, samples corresponding to the "typica", "monophylla" and "echinochila" morphotypes were revealed as three distinct clusters in the NJ phenogram (Fig. 4). Strong BS (100%) was found for the clusters "monophylla" and "typica", but not for "echinochila" (67% BS). On the other hand, samples belonging to the "acicularis" morphotype (only three individuals of one population sampled) split in two distinct groupings. The "acicularis" samples are genetically more similar to the "echinochila" and "monophylla" morphotypes than to the "typica" samples (Fig. 4).

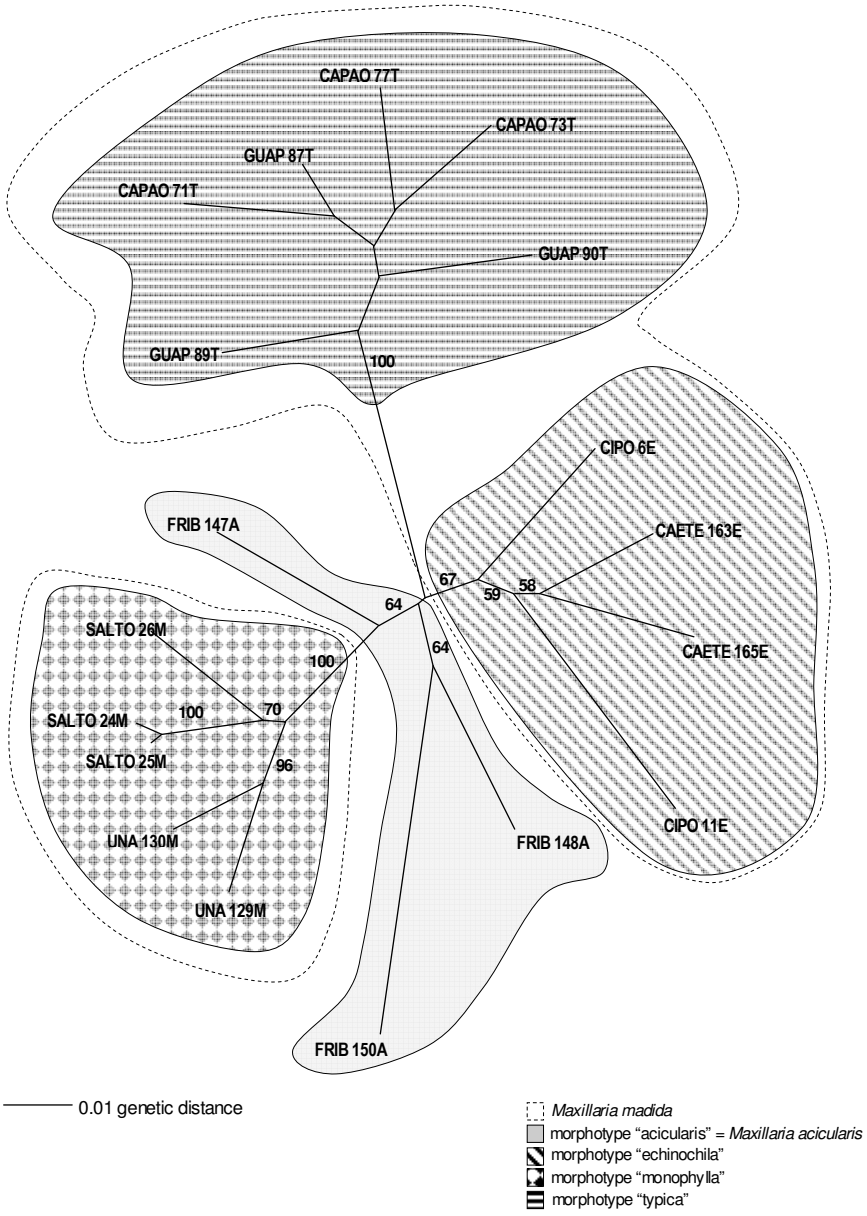


Fig. 4. Neighbor-joining phenogram based on Nei & Li similarity coeficient of the '*Maxillaria acicularis - M. madida*' clade resulting from the pilot data matrix. Sample identity follow Table 2. Morphotypes are indicated by letters at the end of each name: A= "acicularis", E= "echinochila", T= "typica", M= "monophylla".

The MP analysis generated two most parsimonious trees (MPT) of length 431, with a consistency index (CI) of 0.40 and a retention index (RI) of 0.58 (171 potentially parsimoniously informative characters included; total number of characters equals 338). The results of the MP analysis agree with the NJ one (Figs. 4, 5) and the only difference in the topology of the two equally parsimonious trees is the position of sample GUAP90 within the "typica" clade. Clades representing samples currently recognized as *M. acicularis* and *M. madida* are also not supported by the MP analysis. Instead, the morphotypes "echinochila", "monophylla" and "typica" are all depicted as monophyletic groups, with strong BS for "monophylla" and "typica" (72% for "echinochila") (Fig. 5). The morphotype "acicularis" (= *M. acicularis*) was not recovered as a monophyletic group (Fig. 5).

The results of the PCoA analysis generally confirmed the relationships shown by the NJ and MP analyses, with the first two principal coordinates explaining 42.7% of the variation among samples (Fig. 6). The "typica" and "monophylla" morphotypes are, again, easily distinguished from the other samples (Fig. 6). However, there is no clear distinction between the "echinochila" and "acicularis" morphotypes, which cluster together in a third grouping in the scatter plot (Fig. 6). Contrary to the NJ and MP results, the PCoA analysis suggests the "echinochila" plus "acicularis" morphotypes to be more similar to each other than to the "monophylla" one. In general, individuals belonging to the same population grouped together/are phylogenetically more closely related in both NJ/MP trees.

Final analysis

The second, final analyses, considering 56 samples and 18 populations, also do not sustain *M. acicularis* and *M. madida* as two distinct taxa (represented by clusters or clades). The main difference between the pilot and final analyses is that the morphotypes "echinochila", "monophylla" and "typica" do not emerge as monophyletic taxonomic entities.

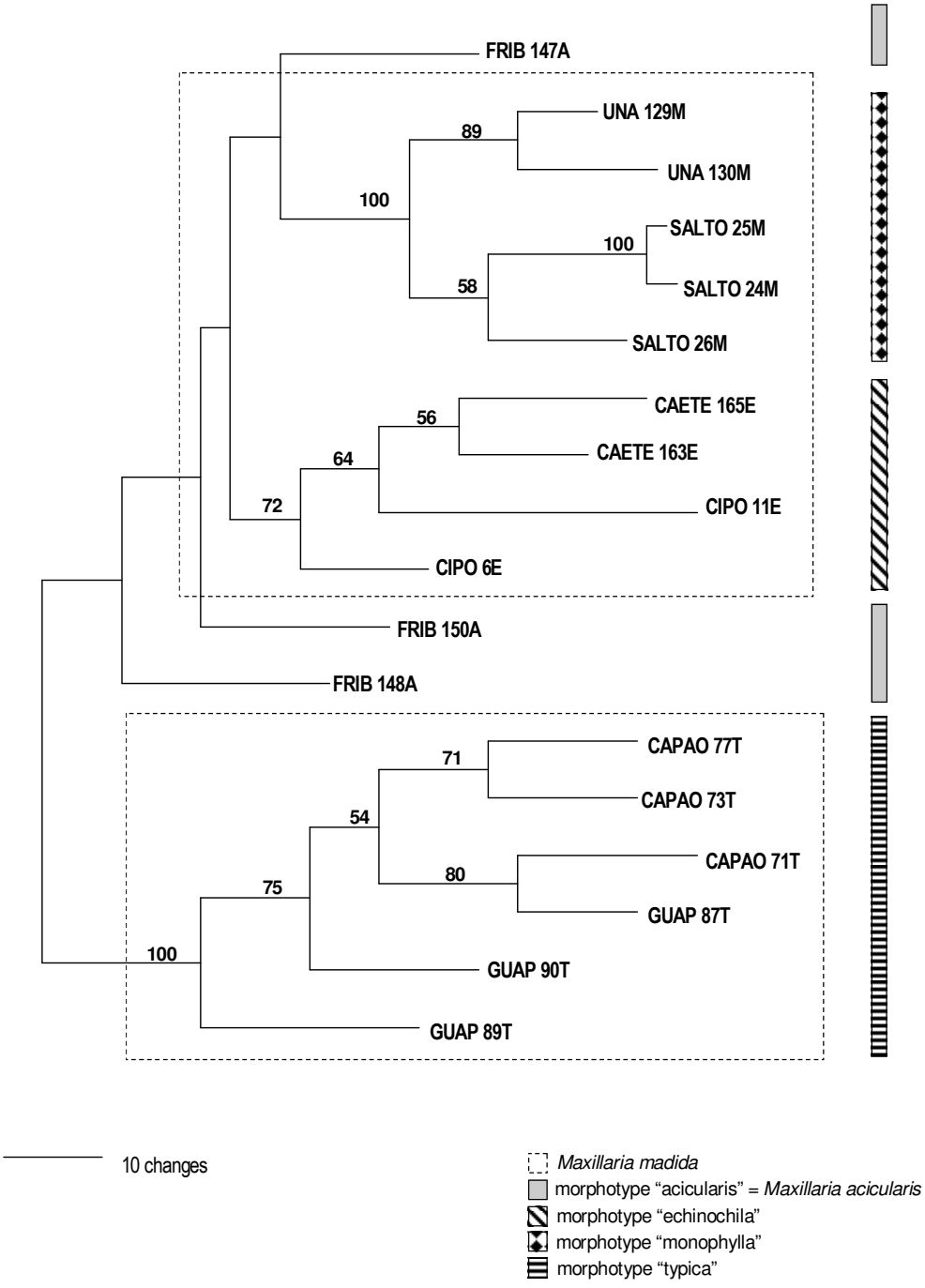


Fig. 5. One of the two most parsimonious trees of the *"Maxillaria acicularis - Maxillaria madida"* clade for the pilot data matrix. Sample identity follow Table 2. Length= 431, CI= 0.40, RI= 0.58. Morphotypes are indicated by letters at the end of each name: A= "acicularis", E= "echinochila", T= "typica", M= "monophylla".

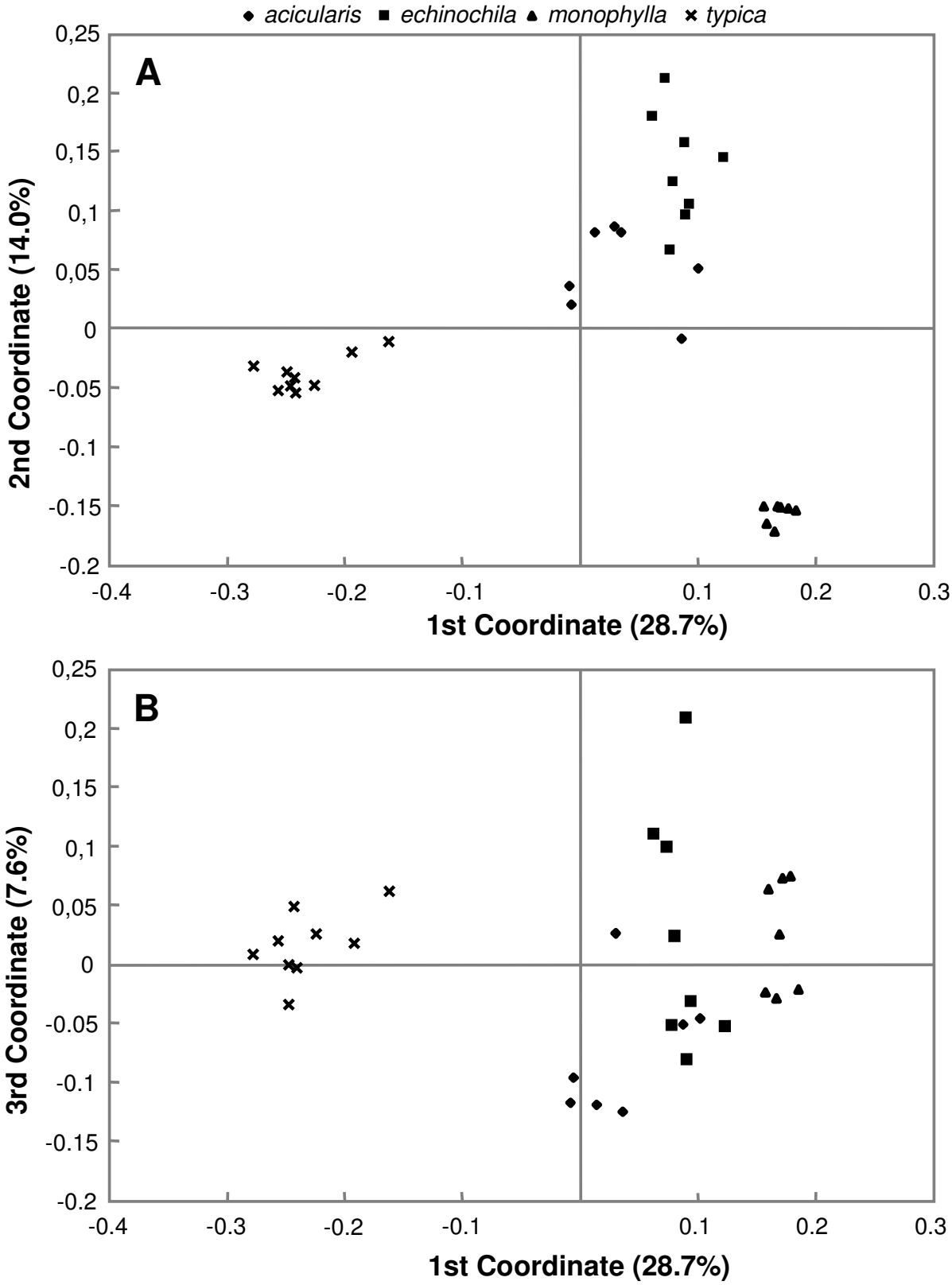


Fig. 6. Principal component analysis (PCoA) of the *"Maxillaria acicularis - Maxillaria madida"* clade for the pilot data matrix. Sample identity follow Table 2. (A) 1st X 2nd components. (B) 1st X 3rd components.

Populations of plants belonging to the morphotype "echinochila", all from the south-central region of the Minas Gerais state in the Serra do Espinhaço mountain range, were not recovered as exclusive clusters, with the exception of the population from Ouro Preto (OUPR; 87% BS), which comprises the southernmost population sampled within this morphotype. According to the NJ results (Fig. 7, cluster I, BS< 50%), samples from Ouro Preto (OUPR) are genetically more similar to samples from Caeté [CAETE] (66 Km from OUPR) and of some individuals sampled from Serra do Cipó [CIPO] (132 Km from OUPR and 66 Km from CAETE). Cluster II (55% BS) comprises samples from Presidente Jucelino [JUCE] and Serra do Cipó [CIPO], which are located 100 Km from each other. Again, the individuals from JUCE and CIPO do not come out as distinct clusters. One sample of JUCE (195) actually is grouped with CIPO169 with 88% of BS. Cluster III contains only samples from CIPO (BS< 50%). The three groups of samples from CIPO represented in clusters I, II and III are from three different localities, corresponding to different sub-populations, within the Serra do Cipó Nacional Park, which has an area of approximately 33,800 ha.

Cluster IV in the NJ phenogram (Fig. 7; no BS) includes all samples of the "monophylla" morphotype from the states of Bahia and NE Minas Gerais and one of the two populations of "acicularis" sampled, also from Bahia state. The samples of "acicularis" from Floresta Azul (FLAZ) comprise a strong supported cluster, which is genetically more similar to the "monophylla" population from Santa Maria do Salto (SALTO), located 174 Km apart. Although samples of "monophylla" from Una (UNA) and Santa Maria do Salto (SALTO), 160 Km apart, are also depicted as separate clusters, these are supported by bootstrap values lower than 50% (Fig. 7). The second population of "acicularis" (from Nova Friburgo; indicated as cluster V and ca. 1.450 Km from the "acicularis" FLAZ population) comes out as the most genetically similar cluster to cluster IV. This population again did not receive BS> 50%; only the cluster formed by individuals FRIB148 and FRIB150 received weak BS support (59%).

Cluster VI is represented in the NJ phenogram by all the samples with an intermediate morphotype between "acicularis" and "echinochila". Two populations, located 132 Km apart, were sampled: Ervália (ERVA) from SE Minas Gerais state, and São Fidélis (SFID), in the northern region of Rio de Janeiro state. Samples from the population SFID constitute a cluster, with two of them as a strongly supported cluster (96% BS).

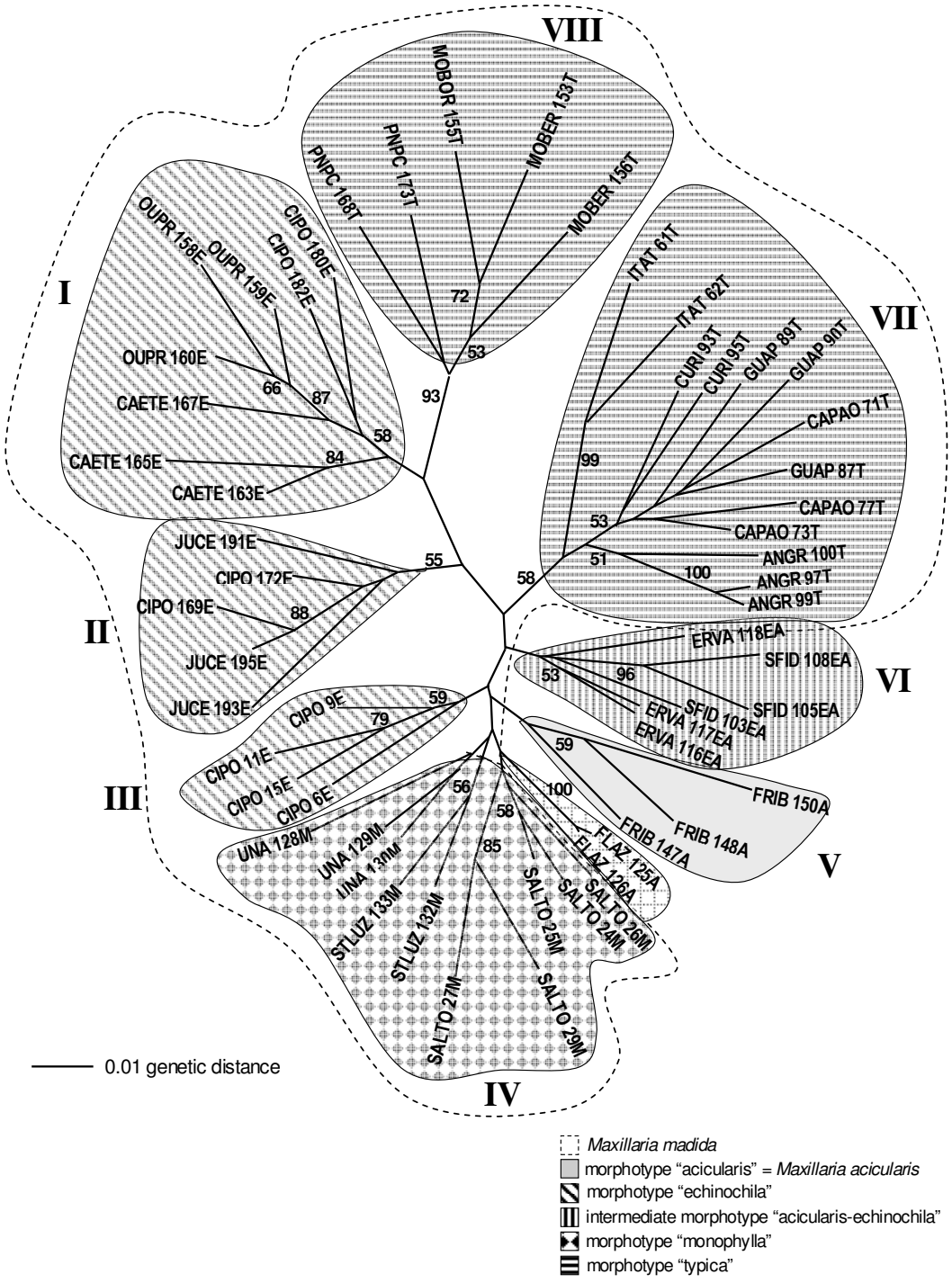


Fig. 7. Neighbor-joining phenogram based on Nei & Li similarity coeficient of the '*Maxillaria acicularis - M. madida*' clade resulting from the final data matrix. Sample identity follow Table 2. Morphotypes are indicated by letters at the end of each name: A= "acicularis", E= "echinochila", EA= "acicularis-echinochila", T= "typica", M= "monophylla".

On the other hand samples from ERVA do not come out as a cluster, since one of its samples is genetically more similar to the ones from SFID than to the other two samples from ERVA (BS< 50%).

The "typica" morphotype is represented in the two last clusters, VII and VIII (Fig. 7). Within cluster VII five populations are clustered with 58% BS. The population of Angra dos Reis [ANGR], from the Rio de Janeiro state (coastal Atlantic rainforest), with 3 individuals, comprises a subcluster with 51% of BS. The two samples from Itatiaia (ITAT), also from Rio de Janeiro state (Serra da Mantiqueira mountain range) and located 64 Km from ANGR, appear as a strongly supported subcluster with 99%BS. The third group within cluster VII (BS of 53%) is represented by samples from São Paulo state in one sub-group (five individuals from populations CAPAO and GUAP from Serra de Paranapiacaba mountain range) and Paraná state in another sub-group (two individuals from CURI from Serra do Mar mountain range). Within this group, the two populations from São Paulo, Capão Bonito [CAPAO] and Guapiara [GUAP], are not distinguished in the NJ phenogram, with one individual of CAPAO indicated as genetically more similar to the ones from GUAP than to others from CAPAO. These two populations are both located in the south-east region of São Paulo state, located only 36 Km apart from each other. The second cluster with "typica" samples received 93% of BS and includes samples from the road SP098 (Moji das Cruzes – Bertioga [MOBER]) in the Serra do Mar mountain range and from the city of Paranapiacaba [PNPC], located in the Serra de Paranapiacaba mountain range. Although samples from CAPAO, GAUP and PNPC all come from the same mountain range, MOBER and PNPC are located, nonetheless, geographically closer to each other than PNPC is from CAPAO and GUAP.

The MP analysis resulted in 29 equally parsimonious trees (length 1127, CI=0.17, RI= 0.51). Relationships supported by the MP analysis are somewhat similar to the NJ final one (Figs. 7, 8). Current definitions of *M. acicularis* ("acicularis" morphotype) and *M. madida* ("echinochila", "monophylla" and "typica" morphotypes) are also not supported as monophyletic groups by the MP analysis (Fig. 8). Populations of "acicularis" are embedded within a clade containing samples belonging to the morphotypes "monophylla", and "typica" + the intermediate populations of "acicularis-echinochila" (Fig. 8).

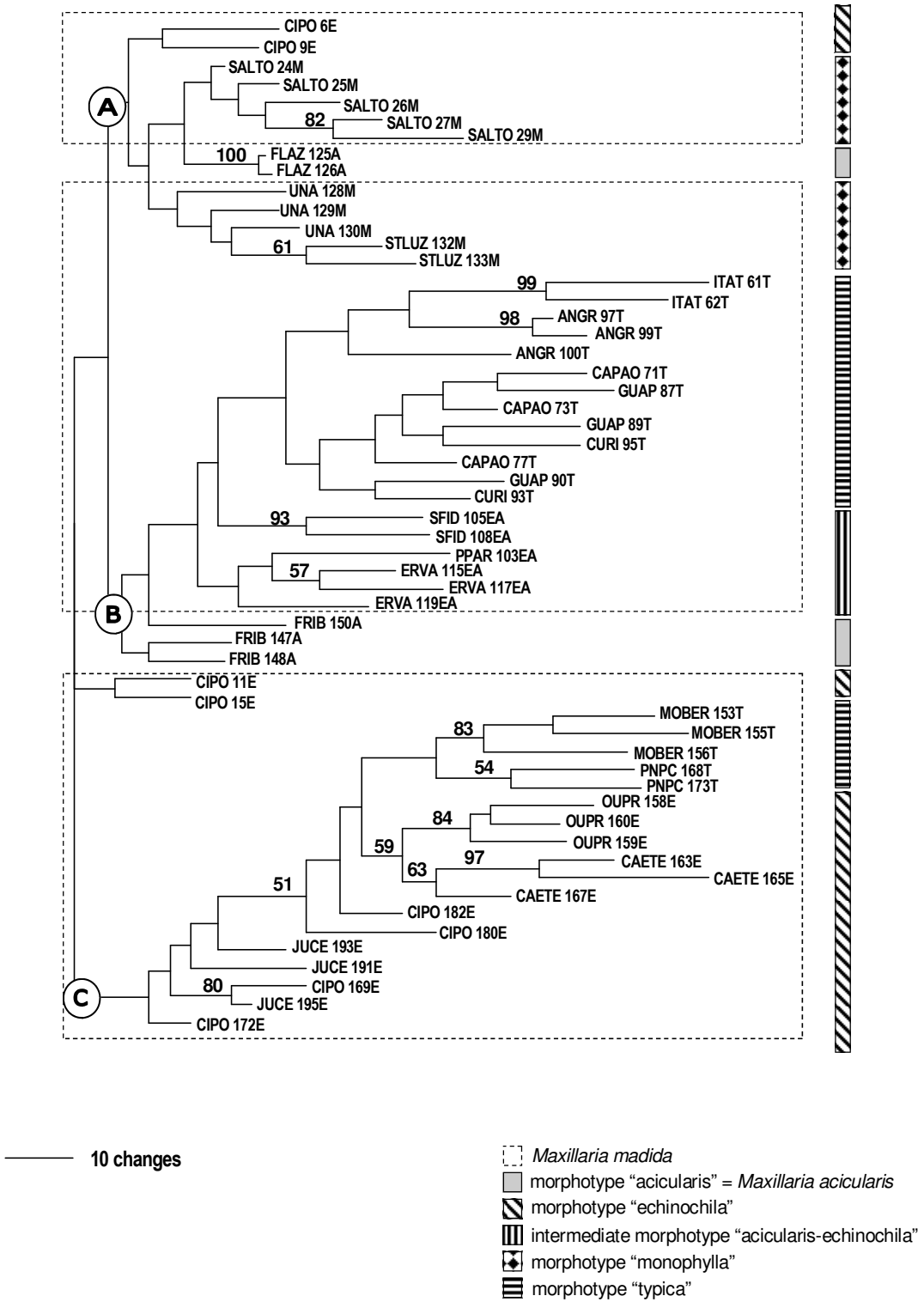


Fig. 8. One of the 29 most parsimonious trees of the *"Maxillaria acicularis - Maxillaria madida"* clade for the final data matrix. Sample identity follow Table 2. Length= 1127, CI= 0.17, RI= 0.51. Morphotypes are indicated by letters at the end of each name: A= "acicularis", E= "echinochila", EA= "acicularis-echinochila", T= "typica", M= "monophylla".

Contrary to the MP pilot study, morphotypes are not depicted as monophyletic groups in the final MP analysis. Cluster VIII ("typica" samples) of the NJ analysis comes out as a monophyletic group (no BS support) in the MP analysis, embedded in a clade with "echinochila" samples from NJ clusters I and II (Fig. 8, clade C). Samples represented in this clade are also suggested as genetically similar by the NJ results (clusters I, II and VIII), but with no BS> 50% for neither NJ nor MP analyses. Two samples of cluster II (CIPO 11E + CIPO 15E) are unplaced in the tree (Fig. 8).

The other samples of "typica" (NJ cluster VII) also comprise a monophyletic group (BS< 50%) embedded within clade B (Fig. 8), which contains samples also of the intermediate morphotype "acicularis-echinochila" and of "acicularis" from Nova Friburgo (FRIB). Samples depicted in clade B were also suggested as genetically similar by the NJ phenogram (Figs. 7, 8). Within clade B, BS values greater than 50% are restricted to populations or groups of individuals within populations: 99% BS for ITAT; 98% for two individuals from ANGR; 57% for two individuals from ERVA; and 93% for two individuals of SFID (Fig. 8).

Clade A (Fig. 8) contains the samples from Bahia state belonging to the morphotypes "acicularis" and "monophylla" plus samples from NE Minas Gerais of "monophylla" (BS< 50%). This clade is represented in the NJ analysis as cluster IV. Within clade C, the population of "monophylla" from SALTO comes out as the sister group of FLAZ (morphotype "acicularis", 100% BS). These two populations are sister to the "monophylla" populations UNA (paraphyletic) and STLUZ (Fig. 8). Two other samples of "echinochila" (CIPO 6E + CIPO 9E) come out as the sister group of the previous described clade comprising a clade denominated as clade A.

PCoA eigenvectors obtained for samples in the final analysis were classified according to morphotypes and habitat (humid x dry) (Figs. 9 and 10, respectively). In accordance with the previous results presented for NJ and MP analyses, *M. acicularis* and *M. madida* are not supported as two separate groups in the PCoA scatter plot (Figs. 9, 10). On Fig. 9A (1st and 2nd coordinates) we can identify a group containing samples of the "typica" morphotype and another comprising most of "echinochila" samples. A third group is represented by the samples of "monophylla" from Bahia and NE Minas Gerais (maximum distance between populations within this group of 174 Km); of "acicularis" from Bahia and

Rio de Janeiro (1450 Km apart); of all intermediate samples of "acicularis-echinochila" from Minas Gerais and Rio de Janeiro (132 Km apart); and by four samples from Minas Gerais belonging to the "echinochila" morphotype (samples from CAETE and CIPO, 66 Km apart). The distinct morphological/geographical groups are less clear on Fig. 9B (1st and 3rd coordinates). Although the "monophylla" and "acicularis-echinochila" groups are easily recognized, with samples from "acicularis" close to "monophylla", the other two morphs recognized in Fig. 9A ("typica" and "echinochila") are not recognized as individual groups in Fig. 9B.

The same relationships are shown in Fig. 10, with samples classified per habitat. In Fig. 10A (1st and 2nd coordinates) we can clearly recognized three groups: a first one belonging to southern humid habitats (Atlantic rainforest within the mountain ranges of Serra do Mar [Paraná, Rio de Janeiro and São Paulo states], Serra de Paranapiacaba [São Paulo state] and Serra da Mantiqueira [Rio de Janeiro state]); a second one representing samples from the dry habitats (Minas Gerais state, Serra do Espinhaço mountain range, "echinochila" morphotype), and a third one containing samples from northern humid areas of Bahia and NE Minas Gerais states (lowland Atlantic rainforest) (Fig. 10A). We also recognize an overlap between some samples from the humid southern Atlantic rainforest and the drier ´campos rupestres´ of Minas Gerais (Fig. 10A). On Fig. 10B (1st and 3rd coordinates) the only clearly recognizable group comprises the northern humid samples from the Atlantic lowland rainforests of Bahia and NE Minas Gerais, including the "monophylla" and "acicularis" morphotypes. The other samples from southern humid and dry areas completely overlap.

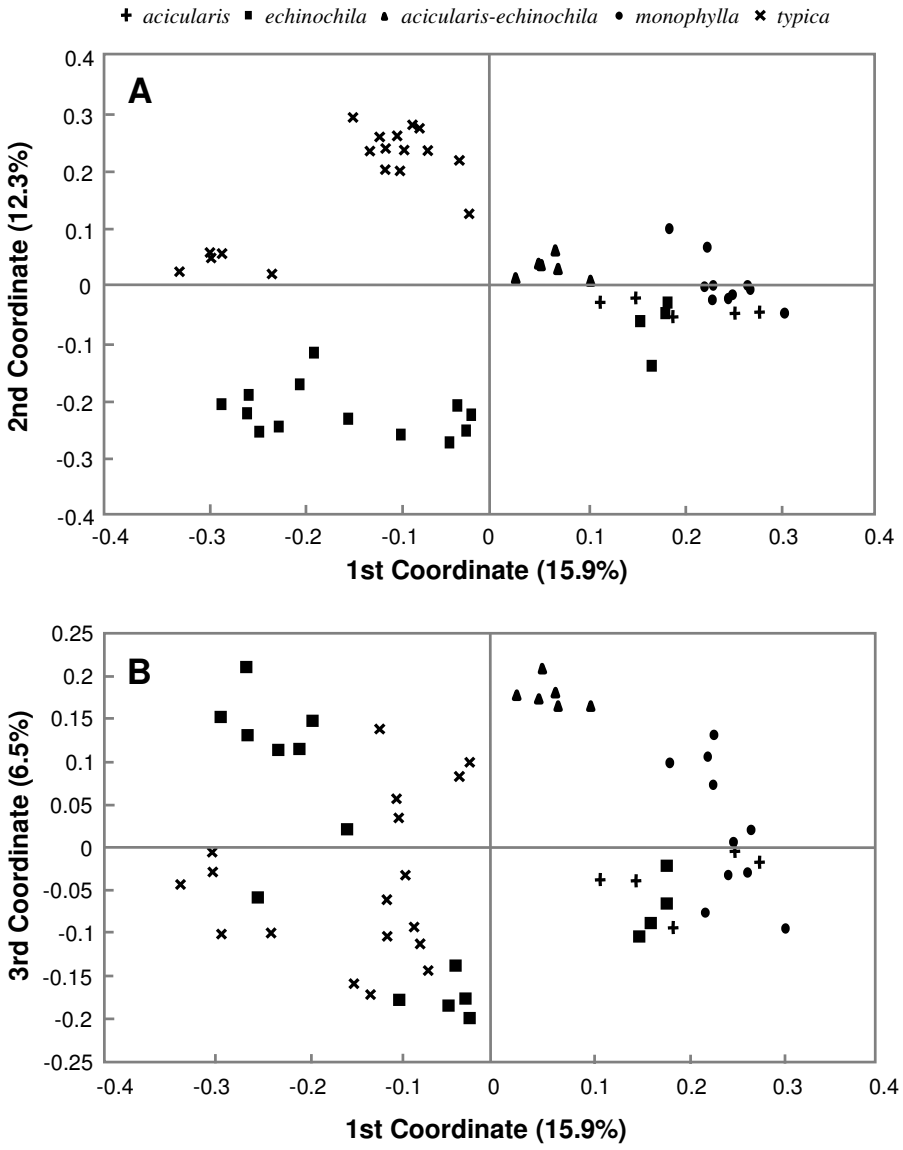


Fig. 9. Principal component analysis (PCoA) of the *"Maxillaria acicularis - Maxillaria madida"* clade for the final data matrix. Sample identity follow Table 2. Samples are classified according to morphotype classification. (A) 1st X 2nd components. (B) 1st X 3rd components.

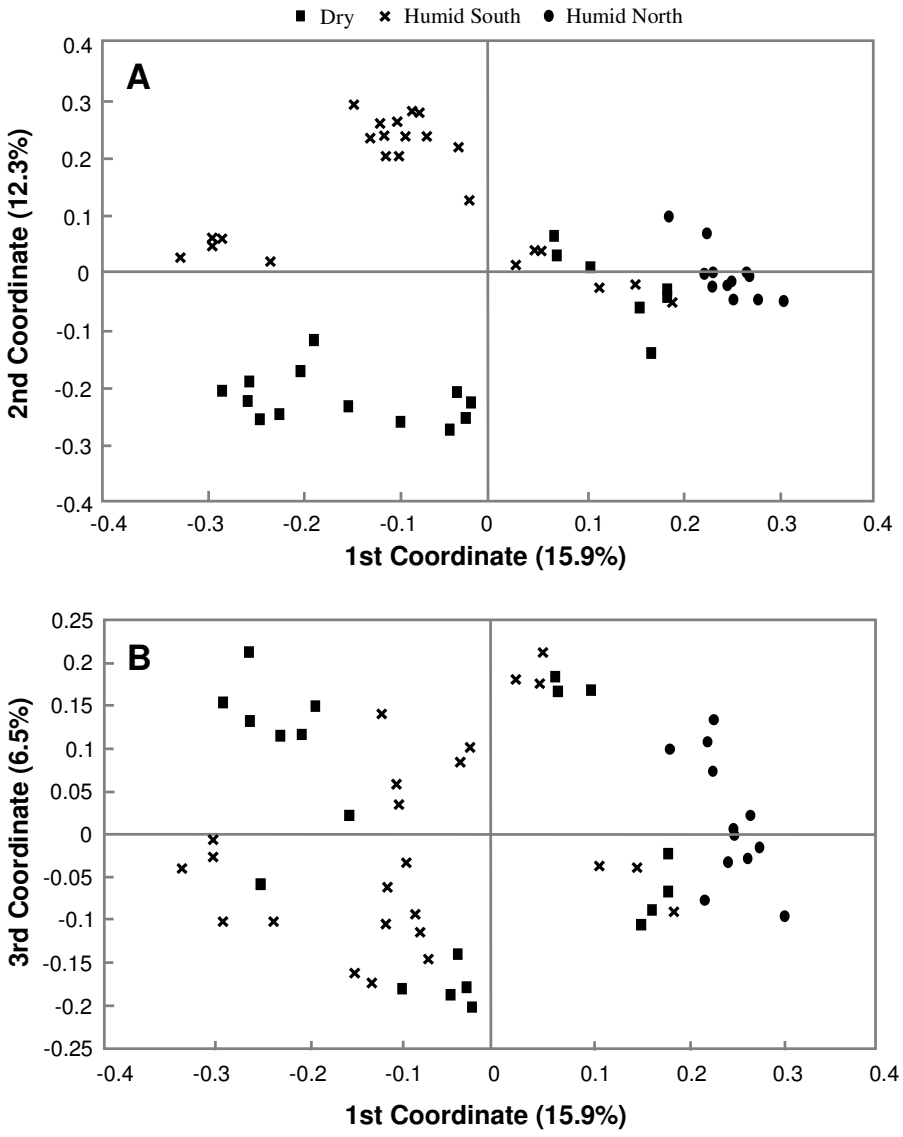


Fig. 10. Principal component analysis (PCoA) of the *"Maxillaria acicularis - Maxillaria madida"* clade for the final data matrix. Sample identity follow Table 2. Samples are classified according to habitat occurrence (A) 1st X 2nd components. (B) 1st X 3rd components.

Discussion

Species delimitation

According to the Genotypic Cluster Species Concept (GCSC, sensu Mallet 1995a) adopted in this study, *Maxillaria acicularis* and *M. madida*, as currently defined, are not supported as distinct genotypic clusters by the obtained AFLP data (Figs. 4-10) and, therefore, cannot be recognized as two different species. Instead, samples of *M. acicularis* ("acicularis" morphotype) from Bahia are genetically more similar to other samples from Bahia belonging to the morphotype "monophylla" of *M. madida* than they are to other samples of "acicularis". The other population of "acicularis" sampled also appears embedded within the *M. madida* samples (Figs. 4-10).

All analyses performed, including the pilot and final data sets, suggest a certain degree of clustering accordingly to vegetative morphology (morphotype classification), especially regarding "echinochila", "monophylla" and "typica" (Figs. 4-10). In the pilot analysis, the NJ and MP trees suggested these three morphotypes as individual clusters/clades with BS values varying from 72 – 100% (Figs. 4, 5). Such distinction is less clear in the PCoA pilot analysis regarding the "echinochila" morphotype, which comes out together in a single group with "acicularis" samples (Fig. 6). Distinction between the "acicularis" and "echinochila" morphotypes can be indeed rather challenging. The "echinochila" morphotype has been generally characterized by its two extremely convolute, straight leaves, while the "acicularis" morphotype is usually recognized as more delicate, undersized plants with two linear leaves and small flowers. However, young delicate plants with very thin, linear leaves, characteristic of the "acicularis" morphotype, are frequently found among plants of "echinochila" in the field. Also, it is common to find in the same population, both extremes of variation, or even populations with intermediate leaf morphology, such as ERVA and SFID. Flower morphology and size also do not covary with leaf variation, since one can find delicate plants with linear leaves but large flowers or the reverse.

The final analyses considering a larger number of individuals and populations were less resolved (Figs. 7-10) probably due to a insufficient number of informative characters, as discussed in the methodological considerations section. Moreover, the blurred genetic boundaries among morphotypes, as suggested by the results of the final analysis, could be

a consequence of the rapid adaptative divergence within-species level due to differential habitat colonization. In this way, populations may not be reproductively isolated or time was insufficient for the accumulation of informative genetic characters and divergent natural selection may still be in the process of driving the accumulation of gene flow barriers between morphotypes at the time of the study (Lexer & Fay 2005). Nonetheless, the analyses carried out with the final data set also indicate some degree of clustering in agreement with morphotype classification (Figs. 7-9).

The morphotype classification proposed in this study was partially supported by the final NJ phenogram (Fig. 7). Three out of eight major clusters (numbered in Fig. 7) contain exclusively "echinochila" samples (clusters I-III), two clusters contain the "typica" samples (VII-VIII), samples of "acicularis" from population FRIB are restricted to cluster V, and all samples with the intermediate morphotype "acicularis-echinochila" are restricted to cluster VI (Fig. 7). The only exception is cluster IV, which contains all samples of "monophylla" from southern Bahia and NE Minas Gerais plus samples of "acicularis" from one population also from southern Bahia (Fig. 7). The results of the MP phylogenetic analysis partially agree with the NJ phenogram and also indicate some correlation between morphotypes classification and phylogenetic history (Fig. 8). The clades represented in the MP tree in agreement with the clusters identified in the NJ analysis are ("monophylla" + "acicularis" samples from FLAZ) [cluster IV], and the "typica" samples, which come out in two clades corresponding to clusters VII and VIII. The "echinochila" clusters I + II as well as cluster VI ("acicularis-echinochila") come out as paraphyletic groups in the MP tree (Fig. 8). As in the NJ phenogram, there is no support for "acicularis" as a distinct group in the MP analysis. The PCoA analysis (1^{st} x 2^{nd} coordinates) support NJ clusters I + II as a single group (representing partially the "echinochila" morphotype) as well as "typica" samples represented in NJ clusters VII and VIII. All the other samples appear in the PCoA scatter plot as a third group, with samples of "monophylla" forming a sub-group within it (Fig. 8).

Besides a correlation between genetic variation and morphology, the data here presented also suggest a correlation between geographic distribution and genetic variation. NJ clusters I-III (morphotype "echinochila") are exclusively from the Serra do Espinhaço mountain range, where plants grow in rocky outcrop formations. NJ clusters I

and II are also depicted as a single cluster in the PCoA scatter plot (Fig. 9), but "echinochila" samples represented in NJ cluster III group together with samples belonging to the "acicularis", "monophylla" and "acicularis-echinochila" morphotypes in the PCoA results (Fig. 9). Samples from the rocky outcrop formations do not constitute a monophyletic group according to the MP results. Instead, most samples are embedded in clade A while samples belonging to NJ cluster III appear in other inconsistent positions within the tree.

All the other samples are from the Atlantic rainforest biome. According to our results, samples from the northern Atlantic rainforest (southern Bahia and NE Minas Gerais) comprise a well defined genetic group, indicated as NJ cluster IV, MP clade C and depicted in the PCoA scatter plots as a single group (Figs. 7-9). On the other hand, samples found in the southern Atlantic rainforest, including the states of Rio de Janeiro, São Paulo and Paraná, do not seem to have a single common origin as suggested by the different clusters/clades in the NJ, MP and PCoA results (Figs. 7-9). Two morphotypes are also found in this region, the "typica" and "acicularis" morphotypes. Although both "typica" and "acicularis" morphotypes are epiphytes found exclusively in the Atlantic rainforest, the "typica" morphotype is mainly restricted to the southern Atlantic rainforest, while populations of the "acicularis" morphotype are spread out from north to south. Distributions of "acicularis" and "typica" however are partially overlapping (Fig. 3), with "typica" being much more widespread than "acicularis". However, our results do not suggest "acicularis" and "typica" to be genetic similar/phylogenetically closely related. In order to understand diversification patterns of these two morphotypes, a larger number of populations and individuals are necessary for an appropriate estimation of genetic diversity and gene flow parameters as well for the development of phylogeographic studies.

Despite the fact the morphotypes "acicularis", "monophylla" and "typica" are found in the same vegetational type, they can be mostly distinguished by morphological characters. The "typica" morphotype can be clearly identified by its two flat, lanceolate leaves, while plants of "monophylla" bear always only one long, cylindrical leaf in each pseudobulb (Fig. 2). The morphotype "acicularis" also has bifoliate pseudobulbs, but

leaves are always aciculate, and plants are more delicate than the other morphotypes (Fig. 2).

The morphotype "echinochila", the only one restricted to the rocky outcrop formations of NE Minas Gerais state (Fig. 3), has been characterized by its two extremely convolute, straight fleshy leaves, but distinction between the "echinochila" and "acicularis" morphotypes can be rather challenging sometimes, especially in young, cultivated specimens, since immature plants of both morphotypes usually have two delicate cylindrical leaves/pseudobulb. Although these two morphotypes seem to be restricted to different habitats, populations of "acicularis" are extremely rare and the possibility of its occurrence in patches of more humid vegetational formations dispersed among dryer habitats can not be excluded. The rocky outcrop formations where the morphotype "echinochila" is found was surrounded by seasonally dry forest before the colonization of Brazil in the 1500´s (Fundação SOS Mata Atlântica 2007).

As mentioned above, flower morphology and size within "acicularis" and "echinochila" do not covary with leaf size and form, since one can find delicate plants with linear leaves but large flowers or the reverse (pers. obs.). This situation is even better illustrated by the fact that two populations of this study had to be classified as of intermediate morphology, the "acicularis-echinochila" samples from Ervália (MG) and São Fidélis (RJ). The representation of these distinct populations characterized by the same intermediate morphology as a single cluster in the final NJ phenogram (Fig. 7) and as phylogenetically closely related species in the final MP tree (Fig. 8) may indicate a common hybrid origin followed by dispersion or a stronger gene flow between such populations. In that case, complementary data on cytogenetics, as well sampling of additional loci and individuals, is necessary to clarify the occurrence of putative barriers to gene flow between such distinct morphotypes.

Maxillariinae orchids are mainly pollinated by meliponini bees, although ants and wasps have also been observed as pollinators within this group (Singer & Koehler 2003). Observations on pollination biology demonstrated that plants belonging to the "echinochila" and "typica" morphotypes comprise food-deceptive orchids pollinated by generalist *Melipona* bees, which remove the pollinaria when exploring the lip surface for resources (S. Koehler, unpubl. data). It is highly probable that the "acicularis" and

"monophylla" morphotypes have the same pollination syndrome, as flower size and morphology are extremely similar to the others and no reward is presented. For that reason, gene flow among populations of different morphotypes is expected, at least in the recent past, when populations were probably larger and more widespread. On the other hand, reproductive isolation by postzygotic mechanisms due to karyological differences has been verified for the European orchid *Anacamptis* that evolved in sympatry and share a common pool of pollinators (Cozzolino et al. 2004). Preliminary karyological studies indicate no evidence of such chromosomal rearrangements among morphotypes (J. Cabral & M. Guerra, pers. comm.). Populations sampled by Cabral and Guerra in common with the present study were CAETE, FRIB, FLAZ, PNPC, SALTO, SFID, STLUZ and UNA. Polyploids and distinctive CMA/DAPI banding patterns have been recorded for the populations of "echinochila" and "acicularis – echinochila" sampled (populations SFID and CAETE) (J. Cabral & M. Guerra, pers. comm.). These findings agree with previous studies that demonstrated polyploids are more fit to colonize harsh and unstable environments as compared to their diploid progenitors, probably due to increased heterozygosis and genic and biochemical flexibility provided by the presence of additional alleles (Stebbins 1985). Nevertheless, the occurrence of polyploids in the population SFID, bearing an intermediate morphotype ("acicularis – echinochila"), cannot be explained by the colonization of habitat with harsh conditions, since this population occurs in a forested area. A hybrid origin with subsequent tetraploidization for this group is possible, as referred above, but its corroboration awaits further studies on cytogenetics and infraspecific phylogenetic patterns of diversity.

The hypothesis of *Maxillaria acicularis* and *M. madida* comprising two species considering the GCSC species concept was refuted by our study according to the results of all analyses performed. However, the results here presented also confirm a correlation between leaf morphology (morphotype classification) and habitat/geographical distribution, as already pointed out by Hoehne (1953) and Onishi (1974). Allopatric taxa that are morphologically distinguishable is a problem to the GCSC and many other species concepts, such as the Biological Species Concept (BSC), the Cohesion Species Concept (CSC), and the Evolutionary Species Concept (EvSC) (Coyne & Orr 2004). Considering the

GCSC speciation definition - "the formation of a genotypic cluster that can overlap without fusing with its sibling" (Mallet 1995a) – only sympatric populations can be considered in the analysis to check for the formations of distinct genotypic clusters. Due to technical problems when generating the AFLP markers, we had to drastically reduce the number of samples in the final analysis, especially concerning the "acicularis" morphotype. Sampling additional populations of "acicularis", especially in sympatric areas with the "typica" morphotype are therefore necessary to clarify the occurrence of genotypic clusters. Also, considering the morphological overlapping between "acicularis" and "echinochila" and that areas of Atlantic rainforest 'sensu lato' existed among localities of rocky outcropping formations before the colonization of Brazil (Fundação SOS Mata Atlântica 2007), it would be important to analyze samples of "acicularis" and "echinochila" that are found geographically close to each other in order to verify genetic isolation between these two morphotypes.

Although information on history is not important for the definition of species according to the GCSC (Mallet 1995a), a phylogenetic analysis based on the maximum parsimony criterion was also performed with the obtained data set. The use of phylogenetic methods for the analysis of restriction data has been largely debatable in the literature (e.g. Robinson & Harris 1999, Felsenstein 2004, Koopman 2005). Koopman (2005) undertook an extensive literature survey that indicated a general congruence of AFLP and ITS tree topologies across a wide range of taxonomic groups. We agree with Koopman (2005) in considering different approaches equally important for the analysis of AFLP markers, especially concerning species delimitation.

Nomenclatural considerations

Based on the data here presented, we propose the recognition of the "*Maxillaria acicularis – M. madida*" clade as a single species, *M. subulata* Lindl. Despite the fact the name *M. subulata* has not been related to the *'Maxillaria acicularis – M. madida'* clade in any recent taxonomic treatment, it belongs to this species complex and comprises the oldest legitimate name available, as verified by the examination of its holotype (K). Such a broad species concept is also supported by the following morphological diagnostic characters: pseudobulbs conical, commonly ridged when mature, bearing two, rarely one, acicular,

cylindrical or lanceolate leaves, commonly longer than 50 mm; ovary-pedicel always shorter than adjacent pseudobulb; flowers smaller related to plant size, with generally a strong fruity fragrance, reddish to light brown coloured, usually spotted, petals oblanceolate with an acute apex, lip varying from 7–21 mm, with a ligulate callus. *Maxillaria subulata* can be distinguished from congeneric species as larger plants, up to 30 cm tall, with ridged pseudobulbs and flowers varying from reddish to brownish.

Although this study suggested mostly low genetic differentiation within *M. subulata*, we suggest that the patterns of morphological variation deserves formal recognition in the form of distinct taxonomic infraspecific categories, which are not genetically cohesive but morphologically/ecologically generally so. Considering the drastic number of natural areas destroyed every year in south-eastern Brazil (Morellato & Haddad 2000), the recognition of morphotypes as formal taxonomic units would increase the chances of conservation of morphological distinct populations within *M. subulata*. In addition, plants of *M. subulata* are commonly cultivated by commercial growers, for whom the formal recognition of morphological variation is of great importance. Lastly, despite the low apparent genetic differentiation among populations of distinct morphotypes, there are good diagnostic characters that allow the characterization of infraspecific categories, the exception being a few populations of "acicularis" and "echinochila" that have intermediate morphology.

The use of infraspecific categories has varied greatly and there is still little uniformity in their adoption today (Stace 1998). Considering that morphological variation within *M. subulata* is restricted partially geographically and ecologically, we have opted for assigning the rank of variety for each distinct morphotype, which shall be formally proposed in the taxonomic revision on the *'Maxillaria madida'* complex.

Key to varieties of Maxillaria subulata *Lindl.*

1. Pseudobulbs bearing always one cylindrical leaf, plants mainly found in southern Bahia, rarely Minas Gerais, in humid Atlantic forest. *M. subulata* var. *monophylla* (Cogn.) Koehler
1. Pseudobulbs bearing two leaves, very rarely one (but then leaves lanceolate), occurring in the rocky outcrop formations of Minas Gerais and in humid and seasonally dry forest of south and south-eastern Brazil. 2

2. Plants restricted to the rocky outcrop formations of Minas Gerais, leaves extremely conduplicate as almost cylindrical and fleshy. *M. subulata* var. *subulata*

2. Plants restricted to seasonally dried and humid Atlantic forest, leaves acicular or lanceolate. 3

3. Plants robust with lanceolate leaves. *M. subulata* var. *madida* (Lindl.) Koehler

3. Plants more delicate, with acicular leaves. *M. subulata* var. *angustifolia* (Cogn.) Koehler

Methodological considerations

Since clusters delimited in the NJ and MP final analyses received very low BS or no support at all, the obtained results should be interpreted with caution. Several studies have pointed out the importance of analysing an appropriate number of loci in order to access the underlying population genetic structure (Hollingsworth & Ennos 2004). Although some studies on species delimitation based on AFLP markers obtained robust inferences based on 0.7 – 5.4 loci/sample (3.4 for this study) (Després et al. 2003, Martínez-Ortega et al. 2004, Smith et al. 2004, Whittemore 2005), others required a much higher number of loci per sample (11.2 - 37.6) and, therefore, of primer combinations (7-11), to achieve high confidence in the obtained results (Qamaruz-Zaman et al. 1998, Koopman et al. 2001, Zhang et al. 2001). This is especially applicable to groups with rapid divergence (Albertson et al. 1999).

Insufficient number of characters (loci) seems to explain, at least partially, the low resolution among and within morphotypes depicted in this study. This problem is easily solved by increasing sample size (both number of loci and individuals/populations sampled (Swofford et al. 1996). On the other hand, some sub-clusters/clades depicted in Figs. 7-8 received strong BS (>85%). Before considering the biological meaning of such groups, it must be noted that small datasets are particularly sensitive to sampling (Felsenstein 2004). Samples might cluster close together not because they are closely related, but because the data set available makes them look closely related. In this way, bootstrapping is inherently less reliable when applied to a smaller data set than to a large one (Felsenstein 2004).

Besides the error generated by the restricted sampling (random error, Swofford et al. 1996), it is also important to consider the possibility of violation of model assumptions in the estimation method used (systematic error, Swofford et al. 1996). Both NJ and MP algorithms assume homology and independence of characters used in the analysis. Robinson & Harris (1999) have pointed out that non-homology between co-migrating bands and non-independence of bands on the AFLP gel are certainly applicable to AFLP data. Such sources of error introduce bias into the similarity estimates. Lamboy (1994) calculated the bias on RAPD bands for a range of possible scenarios varying the number of bands, the number of shared bands and the percentages of false negatives and positives. If it is assumed that the only source of error is that of non-homologous bands at a rate of 5% then the bias values calculated by Lamboy (1994) range from 0.5% to 40%, depending on the number of bands detected and the percentage of shared bands. If the results of Lamboy (1994) are applicable to AFLP data, then errors can have the potential to give inaccurate similarity measures and, therefore, can have a large effect on the clustering of taxa - this is particularly problematic when the distance between nodes is small, since small changes can affect the groups of clusters formed. Again, in this case, branch lengths can be easily improved with the addition of more characters in the analysis.

Parsimony methods have increasingly been used in the analysis of restriction site data, such as AFLPs - although AFLPs are detected as fragments, their statistical behaviour is more like that of restriction sites (Felsenstein 2004). With restriction sites the changes in the individual sites occur in different nucleotides and can be regarded as independent unless the sites overlap - this makes easier to apply statistical methods that require independence (Felsenstein 2004). On the other hand, with restriction fragments there is no simple rule as to whether the gain/loss of a restriction fragment corresponds to the gain/loss of a restriction site. But although parsimony methods can be applied to the analysis of restriction site data, the models for AFLP data are still oversimplified, which create additional sources of noise in the analysis (Felsenstein 2004). The main problem with the statistical justification of MP is that it assumes a low rate of change in all characters. If the tree is short enough, unequal ratios of branch length will not cause inconsistency. Thus, contrary to NJ, parsimony will work well for recently diverged species with unequal rates of evolution if branch lengths are not too long (Felsenstein 2004). The individuals sampled

in this study are likely to be very closely related and, therefore, branch lengths are expected not to vary largely among each other. However, poor sampling can determine the occurrence of unequal branch lengths in the tree, as observed in both NJ and MP trees generated in this study, in which the terminal branches are much more longer when compared to internal ones for several clusters/clades because of the occurrence of alleles restricted to a single or few individuals. Improving loci and individual sampling should avoid the inclusion of a high number of private alleles in the analysis. This is supported by the fact that both NJ and MP pilot analyses have presented a better resolution and more proportionally distributed branch lengths when compared to the final analyses. In the final analysis, where a larger number of individuals was sampled but relatively less characters were included, the estimates of clusters/phylogenetic relationships inferred presented a larger variation (indicated by lower bootstrap support values), suggesting the need to improve loci sampling.

Conclusions

AFLP markers were used to assess genetic relationships among four morphotypes within the *Maxillaria acicularis* – *M. madida* clade, which have been traditionally recognized as two or three (*M. mosenii*) different species with infraspecific varieties. Despite low confidence limits on NJ and MP results, it is clear that the AFLP markers do not support the recognition of *Maxillaria acicularis* and *M. madida* as two species. Instead, a single one, *M. subulata*, with four varieties, should be recognized. Our results also suggest there is little genetic differentiation among morphotypes and, probably, high gene flow among populations, although complementary loci are necessary for a better assessment of ancestral polymorphism and current gene flow. It is possible that rapid adaptative divergence has played an important role in the diversification of *M. subulata*, but this remains to be demonstrated by subsequent studies. Future studies, considering additional loci and complementary markers as well cytogenetic data, would provide a better understanding of genetic diversification both within and among populations within *M. subulata*. Undoubtedly, only with the development of multidisciplinary studies can the mechanisms behind the evolution and diversification of neotropical orchids be fully understood.

References

Albertson RC, Markert JA, Danley PD, Kocher TD. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa.

Applied Biosystems. 2005. AFLP plant mapping protocol. 58 pp. URL: http://docs.appliedbiosystems.com/pebiodocs/04303146.pdf.

Avise JC, Ball RM. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. In: Futuyma DJ, Antonovics J (eds.), *Oxford surveys in evolutionary biology*. Oxford University Press, Oxford, pp: 45-67.

Avise JC. 2004. *Molecular markers, natural history, and evolution.* Chapman & Hall, New York.

Barraclough TG, Savolainen V. 2001. Evolutionary rates and species diversity in flowering plants. *Evolution* 55: 677–683.

Berlocher SH. 1998. Origins: a brief history of research on speciation. In: Howard DJ, Berlocher SH (eds.), Endless forms - species and speciation. Oxford University Press, New York, pp: 3-15.

Butzin F., Senghas K. 1996. Subtribus: Maxillariinae, Erster Band, Teil B. In: Brieger FG, Maatsch R, Senghas K (eds.), *Rudolf Schlechter, Die Orchideen*. Blackwell Wissenschafts-Verlag, Berlin, Wien, pp. 1727-1792.

Carvalho GR, Hauser L. 1999. Molecular markers and the species concept: new techniques to resolve old disputes? *Reviews in Fish Biology and Fisheries* 9: 379-382.

Casgrain P, Legendre P. 1998. *Le Progiciel R, Version* 4.0d0. Montreal, Quebec: Département Des Sciences Biologiques, Université de Montréal.

Cogniaux A. 1904. Orchidaceae III. Maxillarinae. In: *Flora Brasiliensis*, vol. 3(6), von Martius C.F.P., Eichler A.G, Urban I. (eds.). Frid. Fleischer, Lipsiae, pp. 1-78.

Coyne JA, Orr HA 2004. Speciation. Sinauer Associates, Inc. Sunderland, MA.

Cozzolino S., Cafasso D., Pellegrino G., Musacchio A., Widmer A. 2003a. Fine-scale phylogeographical analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation. *Molecular Ecology* 12: 2783-2792.

Cozzolino S., Noce M.E., Musacchio A., Widmer A. 2003b. Variation at a chloroplast minisatellite locus reveals the signature of habitat fragmentation and genetic bottlenecks in the rare orchid *Anacamptis palustris* (Orchidaceae). *American Journal of Botany* 90: 1681-1687.

Cozzolino S, D'Emerico S, Widmer A. 2004. Evidence of reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. *Proccedings of the Royal Society, Series B, Biological Sciences* 271: S259-S262.

Cracraft J. 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: Otte D, Endler JA (eds.), *Speciation and its consequences*. Sinauer Associates, Sunderland, MA, pp: 28-59.

Darwin C. 1859. *The Origin of Species by Means of Natural Selection*. John Murray, London.

Després L., Gielly L., Redoutet B., Taberlet P. 2003. Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. *Molecular Phylogenetics and Evolution* 27: 185–196.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure from small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

El-Rabey HA, Badr A, Schäfer-Pregl R, Martin W, Salamini F. 2002. Speciation and species separation in *Hordeum* L. (Poaceae) resolved by discontinuous molecular markers. *Plant Biology* 4: 567–575.

Felsenstein J. 2004. Inferring phylogenies. Sinauer Associates, Inc. Sunderland, MA.

Fitch WM. 1971. Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* 20: 406–416.

Forrest AD, Hollingsworth ML, Hollingsworth PM, Sydes C, Bateman RM. 2004. Population genetic structure in European populations of *Spiranthes romanzoffiana* set in the context of other genetic studies on orchids. *Heredity* 92: 218-227.

Fundação SOS Mata Atlântica. 2007. Projeto Atlas Mata Atlântica. http:// www. sosmatatlantica. org.br/ index.php?section=atlas&action=atlas.

Goldman DH, Jansen RK, van den Berg C, Leitch IJ, Fay MF, Chase MW. 2004. Molecular and cytological examination of *Calopogon* (Orchidaceae, Epidendroideae): circumscription, phylogeny, polyploidy, and possible hybrid speciation. *American Journal of Botany* 91: 707-723.

Gower JC. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika* 53: 325─338.

Gustafsson S., Lönn M. 2003. Genetic differentiation and habitat preference of flowering-time variants within *Gymnadenia conopsea*. *Heredity* 91: 284-292.

Harris SA, Robinson J. 1994. Preservation of Tropical Plant Material for Molecular Analyses. In: Adams RP, Miller JS, Golenberg EM, Adams JE (eds.), *Conservation of Plant Genes II. Monographs in Systematic Botany from the Missouri Botanical Garden 48*. Missouri Botanical Garden, St. Louis, pp. 83-92.

Harris SA. 1999. RAPD in systematics - a useful methodology? In: Hollingsworth PM, Bateman RM, Gornall RJ (eds.) *Molecular systematics, plant and evolution*. Taylor and Francis, London, pp: 221-228.

Hedrén M., Fay MF, Chase MW. 2001. Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). *American Journal of Botany* 88: 1868-1880.

Hedrén M. 2003. Plastid DNA variation in the *Dactylorhiza incarnata/maculata* polyploid complex and the origin of allotetraploid *D. sphagnicola* (Orchidaceae). *Molecular Ecology* 12: 2669-2680.

Hennig W. 1950. *Grundzüge einer Theorie der phylogenetischen Systematik*. Aufbau, Berlin.

Hennig W. 1966. *Phylogenetic systematics.* University of Illinois Press, Urbana, IL.

Hillis DM. 1994. Homology in molecular biology. In: Hall B.K. (ed.), *Homology, the Hierarchical Basis of Comparative Biology*. Academic Press, San Diego, pp. 339-368.

Hoehne FC. 1947. Reajustamento de algumas espécies de Maxillarias do Brasil, com a criação de dois novos gêneros para elas. *Arquivos de Botânica do Estado de São Paulo* 2: 65–73.

Hoehne FC. 1953. Orchidaceas. In: *Flora Brasilica* 10 (12, 7). Secretaria da Agricultura, São Paulo, pp. 223-341

Hollingsworth PM, Ennos RA. 2004. Neighbour joining trees, dominant markers and population genetic structure. *Heredity* 92: 490-498.

Howard DJ, Berlocher SH. 1998. *Endless forms species and speciation.* Oxford University Press Oxford, United Kingdom

Indsto JO, Weston PH, Clements MA, Whelan RJ. 2004. Highly sensitive DNA fingerprinting of orchid pollinaria remnants using AFLP. *Australian Systematic Botany* 18: 207-213.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. *Bulletin de la Societe Vaudoise de Sciences Naturelles* 44: 223–270.

Kardolus JP, van Eck HJ, van den Berg RG. 1998. The potential of AFLPs in biosystematics: a first application in *Solanum* taxonomy (Solanaceae). *Plant Systematics and Evolution* 210: 87-103.

Koopman WJM, Zevenbergen MJ, van den Berg RG. 2001. Species relationship in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. *American Journal of Botany* 88: 1881-1887.

Koopman WJM. 2005. Phylogenetic signal in AFLP data sets. *Systematic Biology* 54: 197-217

Lamboy W. 1994. Computing genetic distance similarity coefficients from RAPD data: the effects of PCR artifacts. *PCR methods and applications* 4: 31-37.

Leaché AD, Reeder TW. 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. *Systematic Biology* 51: 44-68.

Lexer C, Fay MF. 2005. Adaption to environmental stress: a rare or frequent driver of speciation? *Journal of Evolutionary Biology* 18: 893-900.

Mace ES, Lester RN, Gebhardt CG. 1999. AFLP analysis of genetic relationships among the cultivated eggplant, *Solanum melongena* L. and wild relatives (Solanaceae). *Theoretical and Applied Genetics* 99: 626–633.

Mallet, J. 1995a. A species definition for the Modern Synthesis. *Trends in Ecology and Evolution* 10: 294-299.

Mallet J. 1995b. Reply from J. Mallet. *Trends in Ecology and Evolution* 10: 490-491.

Mallet, J. 1997. What are species? (Review of "Species, the Units of Biodiversity", edited by M.F.

Claridge, H.A. Dawah, and M.R. Wilson.). *Trends in Ecology and Evolution* 12: 453-454.

Mallet J. 2006. Species concepts. In: Fox CW, Wolf JB (eds.), *Evolutionary genetics: concepts and case studies*. Oxford University Press, Oxford, pp: 367-373.

Martínez-Ortega MM, Delgado L, Albach DC, Elena-Rosselló JA, Rico E. 2004. Species boundaries and phylogeographic patterns in cryptic taxa inferred from AFLP markers: *Veronica* subgen. *Pentasepalae* (Scrophulariaceae) in the Western Mediterranean. *Systematic Botany* 29: 965-986.

Mayr E. 1942. *Systematics and the origin of species*. Columbia University Press, New York.

Mayr E. 1959. Where are we? *Cold Spring Harb. Symp. Quant. Biology* 24: 1-14.

Mayr E. 1982. *The growth of biological thought.* Harvard University press, Cambridge, MA.

McKitrick MC, Zink RM. 1988. Species concepts in ornithology. *Condor* 90: 1-14.

Meier R, Willmann R. 2000. The Hennigian species concept. In: Wheeler QD, Meier R (eds.), *Species concepts and phylogenetic theory, a debate.* Columbia University Press, New York, pp: 30-43.

Morellato LP, Haddad CFB. 2000. Introduction: The Brazilian Atlantic Forest. *Biotropica* 32: 786-792.

Mueller U., Wolfenbarger L. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* 14: 389–394.

Nei M., Li W.-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, USA, 76: 5269-5273.

Onishi E. 1974. Sobre o polimorfismo do grupo *Maxillaria madida* (Orchidaceae, Maxillarinae). Tese de Doutorado. Escola Superior de Agronomia Luis de Queiroz, USP, São Paulo.

Pabst GFJ, Dungs F. 1977. *Orchidaceae Brasiliensis II*. Brücke–Verlag Kurt Schmersow, Hildesheim.

Paterson HEH. 1985. The recognition concept of species. In: Vrba ES (ed.), Species and speciation S. Vrba, ed.). Transvaal Museum Monograph No. 4, Transvaal Museum, Pretoria, pp: 21-29.

Poulton EB. 1908. What is a species? *Essays on evolution*. Oxford, Claredon Press, Oxford, pp: 46-94.

Qamaruz-Zaman F, Fay MF, Parker J, Chase MW. 1998. The use of AFLP fingerprinting in conservation genetics: a case study of *Orchis simia* (Orchidaceae). *Lindleyana* 13: 125-133.

Robinson JP, Harris SA. 1999. Amplified Fragment Length Polymorphisms and microsatellites: a phylogenetic perspective. In: Gillet EM (ed.), *Final Compedium of the Research Project Development, optimisation and validation of molecular tools for assessment of biodiversity in forest trees in the European Uniion.* DGXII Biotechnology FW IV Research Programme Molecular Tools for Biodiversity. URL: http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.doc.

Russell JR, Weber JC, Booth A, Powell W, Sotelo-Montes C, Dawson IK. 1999. Genetic variation of *Calycophyllum spruceanum* in the Peruvian Amazon Basin, revealed by amplified fragment length polymorphism (AFLP) analysis. *Molecular Ecology* 8: 199-204.

Shaw KL. 2001. The genealogical view of speciation. *Journal of Evolutionary Biology* 14: 880-882.

Simpson GG. 1951. The species concept. *Evolution* 5: 285-298.

Singer RB, Koehler S. 2003. Toward a phylogeny of Maxillariinae orchids: multidisciplinary studies with emphasis on Brazilian species. *Lankesteriana* 7: 57-60.

Sites JW, Crandall KA. 1997. Testing species boundaries in biodiversity studies. *Conservation Biology* 11: 1289-1297.

Smith S D, Cowan RS, Gregg KB, Chase MW, Maxted N, Fay MF. 2004. Genetic discontinuities among populations of *Cleistes* (Orchidaceae, Vanilloideae) in North America. *Botanical Journal of the Linnean Society* 145: 87-95.

Sneath PHA, Sokal RR. 1973. *Numerical taxonomy*. San Francisco: W. H. Freeman.

Sokal RR, Crovello TJ. 1970. The biological species concept: a critical evaluation. *American Naturalist* 104: 127-153.

Squirrell J, Hollingsworth PM, Bateman RM, Tebbitt MC, Hollingsworth ML 2002. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). *Molecular Ecology*11: 1957-1964.

Stace CA. 1998. Plant taxonomy and biosystematics. 2nd Edition. New York: Chapman and Hall Inc.

Stebbins GL (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Gardens* 72: 824–832.

Swofford DL, Olsen, GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular Systematics. 2nd Ed.* Sinauer Associates, Sunderland, MA, pp: 407-514.

Swofford DL. 2000. *PAUP*: phylogenetic analysis using parsimony (* and other methods), Version* 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Templeton AR. 1989. The meaning of species and speciation: a genetic perspective. In: Otte D, Endler JA (eds.), *Speciation and its consequences.* Sinauer Associates, Sunderland, MA, pp: 3-27.

Van der Pijl L, Dodson CH. 1966. Orchid Flowers, their pollination and evolution. Coral Gables, Florida: University of Miami Press.

Van Valen L. 1976. Ecological species, multispecies and oaks. *Taxon* 25: 233-239.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.

Wallace LE. 2003a Molecular evidence for allopolyploid speciation and recurrent origins in *Platanthera huronensis* (Orchidaceae). *International Journal of Plant Sciences* 164: 907-916.

Wallace LE. 2003b. An evaluation of taxonomic boundaries in *Platanthera dilatata* (Orchidaceae) based on morphological and molecular variation. *Rhodora* 105: 322-336.

Whittemore AT. 2005. Genetic structure, lack of introgression, and taxonomic status in the *Celtis laevigata - C. reticulata* complex (Cannabaceae). *Systematic Botany* 30: 809-817.

Whitten WM, Williams NH, Chase MW. 2000. Subtribal and generic relationships of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. *American Journal of Botany* 87: 1842–1856.

Wiley EO. 1978. The evolutionary species concept reconsidered. *Systematic Zoology* 27: 17-26.

Young JE, Crother BI. 2001. Allozyme evidence for the separation of *Rana areolata* and *Rana capita* and for the resurrection of *Rana sevosa*. *Copeia* 2001: 382-388.

Zhang L-B, Comes HP, Kadereit JW. 2001. Phylogeny and quaternary history of the European montane/alpine endemic *Soldanella* (Primulaceae) based on ITS and AFLP variation. *American Journal of Botany* 88: 2331-2345.

Acknowledgements

We are much indebted to the institutions which provided specimens for study as well for all the help of their staff: Instituto de Botânica de São Paulo (E. Catharino, F. de Barros, R. Peres and H. Michelan) and Escola Superior de Agricultura "Luiz de Queiroz" (E. Ann–Veasey, G. Oliveira, J. Pontes). We sincerely thank A. Midori and R. Lourenço for the drawings; C. Verola, F. Pinheiro, J. Aranha, L. Lledó, R. Custódio, R. Cowan, R. Smith, R. Singer, R. Gonçalves and the Jodrell staff for all the lab assistance and/or help on fieldwork and C. Lexer, C. Palma, F. Pinheiro, J. Joseph and T. Barbara for all valuable discussions. This work was supported by funding form Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to S. Koehler (02/02161–7) and M.C.E. Amaral (02/02162–3) and by a Prance Fellowship in Neotropical Botany from the Royal Botanical Gardens Kew to S. Koehler.

3

SINOPSE TAXONÔMICA DO COMPLEXO *'MAXILLARIA MADIDA'*

Synopsis of the Neotropical *'Maxillaria madida'* Complex (Maxillariinae, Orchidaceae)

Samantha Koehler [1,2*], Rodrigo Bustos Singer[3], Maria do Carmo E. Amaral[2]

[1] Pós–graduação em Biologia Vegetal and

[2] Departamento de Botânica, IB, Universidade Estadual de Campinas, Campinas, SP, 13083–970, Brazil

[3] Departamento de Botânica, IB, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91501–970, Brazil

Key words: taxonomy, systematics, orchids, Brazil, South America, Central America

Abstract

A synopsis of *'Maxillaria madida'* species complex (tribe Cymbideae, subtribe Maxillariinae) from the Neotropics is presented. Ten species are recognized: *M. cepula, M. echiniphyta, M. ferdinandiana, M. nardoides, M. neowiedii, M. pacholskii, M. pumila, M. subulata* (with four varieties), *M. uncata* and *M. vernicosa.* Identification keys, diagnostic characters, descriptions with complete taxonomic synonymy, line drawings and distribution maps are presented.

Resumo

Este estudo apresenta uma sinopse taxonômica para o clado de orquídeas neotropicias denominado complexo *'Maxillaria madida'* pertencente à tribo Cymbideae, subtribo Maxillariinae. Dez espécies são reconhecidas:: *M. cepula, M. echiniphyta, M. ferdinandiana, M. nardoides, M. neowiedii, M. pacholskii, M. pumila, M. subulata* (com quatro variedades propostas), *M. uncata* e *M. vernicosa*. Chaves de identificação, caracteres diagnósticos, descrições com sinonímias taxonômicas, ilustrações e mapas de distribuição são apresentados.

Introduction

Phylogenetic studies based on sequence data from nuclear and chloroplast DNA indicates the *'Maxillaria madida'* complex comprises a monophyletic group embedded in the broad Neotropical orchid genus *Maxillaria* (subtribe Maxillariinae; Whitten et al., unpubl. res.). This clade comprises species traditionally recognized in two groups with distinct geographical distributions: the *M. uncata* alliance occurring from southern Mexico to Bolivia (Pabst & Dungs 1977; Carnevali & Atwood, unpubl. res.) and plants currently known as belonging to the *M. madida - M. pumila - M. paulistana - M. neowiedii* alliances (Pabst & Dungs 1977). This latter group is restricted to south-eastern South America, ranging from south of Bahia state in Brazil to northern Argentina (Hoehne 1953, Pabst & Dungs 1977). Floral morphology as well as vegetative architecture are quite conservative in the *'Maxillaria madida'* complex. These plants are relatively small compared to other *Maxillaria* species and commonly bear fleshy to coriaceous leaves, short one-flowered inflorescences and white-yellowish, reddish-purple or light brown rewardless flowers, generally with a conspicuous shiny spot in the midlobe lip. Also, the southeastern Brazilian species of this group usually have roots with conspicuous annular thickenings.

Although plants are easily recognizable as belonging to this clade, species identification comprised a challenging task due to the overlapping of many floral and vegetative characters, especially leaf shape and lip morphology. The most complete taxonomic treatment to include Brazilian species belonging to the *'Maxillaria madida'* complex was undertaken by Hoehne (1953) in which the author recognized 31 taxa (20 species, 11 varieties). Later, Pabst & Dungs (1977) published a list of Brazilian species of orchids including 23 species currently known to belong to this group. These species were divided into four alliances according to the number and shape of the leaves. Hoehne and Pabst & Dungs' species concepts were quite distinct, causing some confusion on the number of taxa to be recognized for this group. While Pabst & Dungs (1977) based their classification mainly on vegetative characters and had a more lumper view of species concepts, Hoehne (1953) complemented his descriptions with plenty of reproductive characters, especially shape of perianth segments and lip morphology, and, as a consequence of the highly variable nature of such characters, proposed the recognition of a much higher number of taxa, several of them being varieties. Despite the differences on

classifications, Hoehne (1953) and Pabst & Dungs (1977) both failed to define species limits. This was probably due to the difficult access to type material as well to the continuously varying nature of morphological characters across most populations within this group.

With the purpose of defining taxonomic units within this morphologically complex group, a phylogenetic study based on nuclear and chloroplast sequence data has been developed for the *'Maxillaria madida'* complex, including all morphological and geographical extremes of variation (Koehler et al., unpubl. res.; Cap. 1). The sequence data gathered combined with a revision of morphological characters allowed the delimitation of 10 species for this group. Later, a complementary study based on AFLP markers was undertaken in order to assess species limits in the sub-clade *'Maxillaria acicularis – M. madida'*. The results indicate a single species with four varieties should be recognized for this complex, according to genetic data and diagnostic morphological characters (Koehler et al., unpubl. data; Cap. 3).

The goal of the present study is to provide a synopsis for the species belonging to the *'Maxillaria madida'* complex in the light of the results obtained by previous studies based on molecular and morphological data (S. Koehler et al., unpubl. data). Identification keys, diagnostic characters, descriptions, distribution maps, and line drawings are provided as well complete taxonomic synonymy are presented.

Materials and methods

Morphology

We based morphological studies mainly on live specimens collected and cultivated by the authors or in cultivation at the "Jardim Botânico de São Paulo" and "Escola Superior de Agricultura Luiz de Queiroz", as well as specimens from F, FLAS, HB, K, MO, NY, OUPR, R, RB, RENZ, SEL, SP, UEC, and US. In addition, the herbaria AMES, BM, BR, HBG, LE, P, S, and W were consulted for nomenclatural types. Despite the importance of these collections, many type specimens could not be located. The two main reasons for that is because they were probably never deposited, as registered for Barbosa Rodrigues' species (Mori & Ferreira 1987), or because they were destroyed, such as the important

collections of R. Schlechter and Kraenzlin at B (Butzin 1978, 1980, 1981, 1983)[7]. Vouchers of all cultivated material were deposited at ESA, SP, and UEC.

Plant structures were analyzed in a stereomicroscope and selected samples were observed in a low vacuum scanning electron microscope (SEM). For the SEM study, samples were collected from live plants and fixed in formaldehyde-acetic acid-50% ethanol (FAA 50). The samples were then dehydrated in a graded ethanol series, critical-point dried with CO_2, and coated in a vacuum with gold-palladium. Plant segments were mounted on aluminium stubs with double-sided sticky, carbon tabs and examined by means of back-scattered electron imaging using a Jeol 5800 LV scanning electron microscope at 10 KV. Drawings were made with the help of a camera lucida attached to a stereomicroscope.

Results

Morphology

The *'Maxillaria madida'* complex comprises plants with sympodial growth, ranging approximately from 1-30 cm high. Species generally grow as epiphytes in the seasonally dry and wet forests of the Amazonian region and south-eastern South America or, more rarely, in rock outcropping formations known as *campos rupestres* in south-eastern Brazil. The roots are white, cylindrical, smooth, with a well-developed velamen and, in the southeastern Brazilian species, conspicuous annular root thickenings are commonly observed (Fig. 1A). The rhizome is rigid, very short to rarely elongate, mostly in *M. uncata*, generally covered by several dry bracts.

The pseudobulbs are aggregate, erect, and made up of a single, thick internode commonly bearing several papery sheaths at the base and one or two, rarely up to four leaves at the apex. The number, shape and size of leaves provide the most important diagnostic characters to distinguish species within this group, the exceptions being *M. subulata* and *M. neowiedii*, which have extremely polymorphic leaves.

---

[7] As ilustrações originais de Barbosa Rodrigues também nunca foram publicadas, apesar de citadas nas descrições originais das espécies. Cópias em nanquim e aquarela dos desenhos originais foram publicadas, respectivamente, por Cogniaux (1904) e Sprunger et al. (1996).

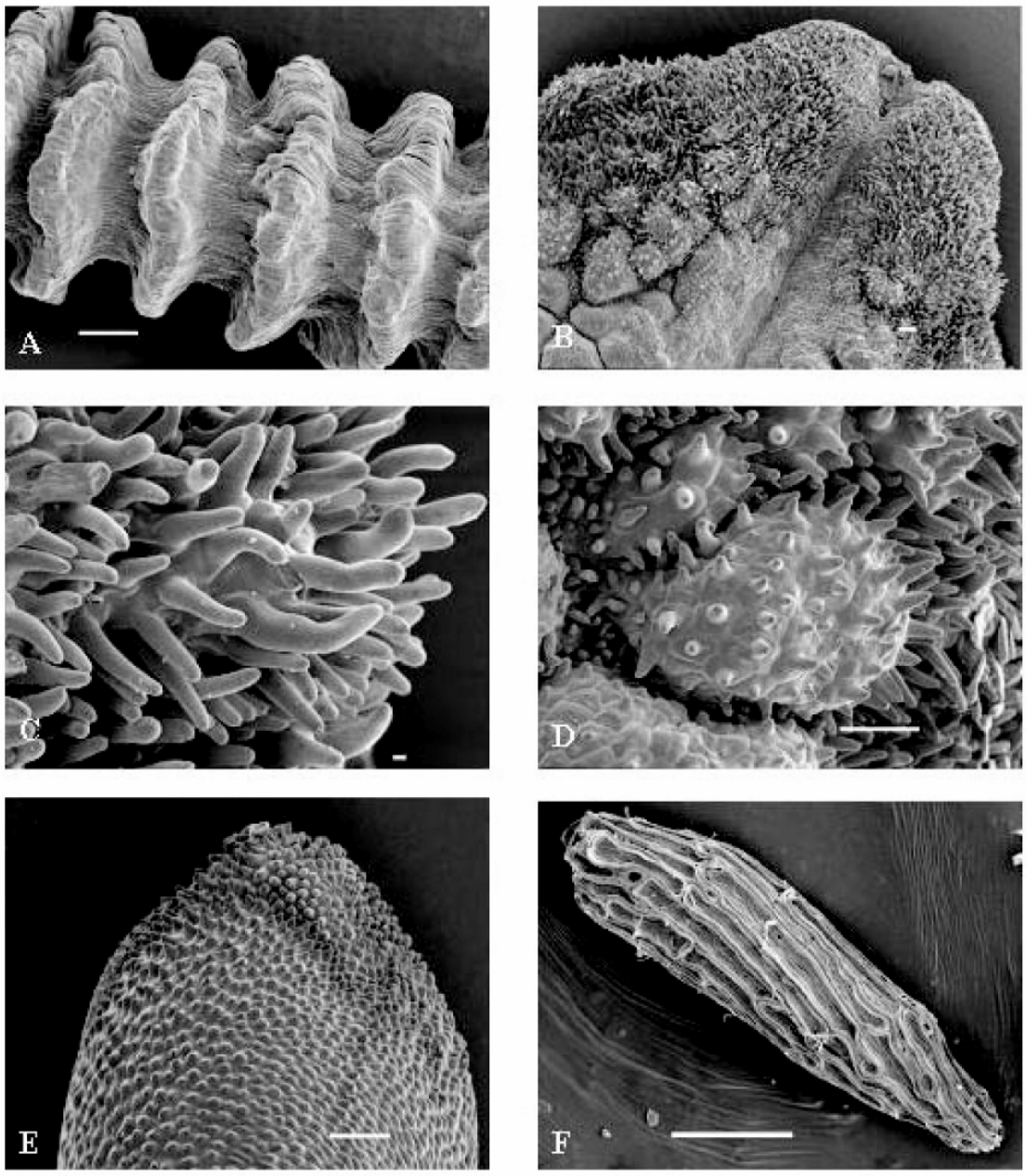


Fig. 1. *Maxillaria neowiedii*. (A) Ringed root (200x, scale 100 µm). *Maxillaria subulata* var. *madida.* (B) Overview of midlobe region (60X, scale 100 µm). (C) Lip trichomes (430X, scale 10 µm). (D) Lip trichomes and papillae (270X, scale 10 µm). (E) Anther cap (200X, scale 100 µm). *Maxillaria neowiedii.* (F) Seed (300X, scale 100 µm).

Nevertheless, these species can be easily identified by other morphological characters, such as plant size, length of the ovary and pedicel in relation to the adjacent pseudobulb and shape of petals. The leaves are always sessile, erect to twisted, flat or conduplicate, sometimes cylindrical, and coriaceous to fleshy, more rarely membranaceous.

Inflorescences vary from one to many, generally two on each side of a pseudobulb. They are extremely reduced, one-flowered, and arise laterally from the base of the pseudobulbs. The short peduncle is always erect and covered by several papery dry, brownish bracts. These are concave, oblong to obovate, acuminate and truncate at the base. The flowers are zygomorphic and resupinate (Fig. 2A) and the pedicel is always subtended by a bract similar in form to the ones from the inflorescence, but usually smaller. Most species are not fragrant or produce very subtle fragrances, the exception being some varieties of *Maxillaria subulata* which have a very strong fruity smell and *M. cepula*, with its "baby-powder like" smelling flowers. The sepals are three, usually slightly larger than the petals, and dissimilar, the dorsal one oblong to obovate, the lateral ones ovate to triangular, rounded and apiculate at the apex, always truncate at the base. The exception are *M. echiniphyta*, *M. pacholskii* and *M. uncata* which have elongate perianth segments, varying from oblong to lanceolate. The two lateral sepals are parallel to each other or divergent and united to the column foot, generally forming an inconspicuous spur. The two lateral petals are always oblique, oblong to oblanceolate, the apex rounded to acute, commonly apiculate, the base attenuate to truncate. The lip varies from lanceolate to obovate, attenuate to cuneate at the apex, entire to slightly 3-lobed in the upper half, always callous, and articulated to the column foot. It is commonly recurved, although in some populations of *M. neowiedii* it is straight or just slightly recurved, glabrous or with conical obpyriform or viliform papilae (Fig. 1B-D; Davies & Turner 2004). The lateral lobes are erect and involute and the midlobe usually has undulate to serrulate margins. In most species, the midlobe bears a conspicuous shiny purple to dark red spot[8].

[8] Ver fotos coloridas das espécies no Anexo 5.

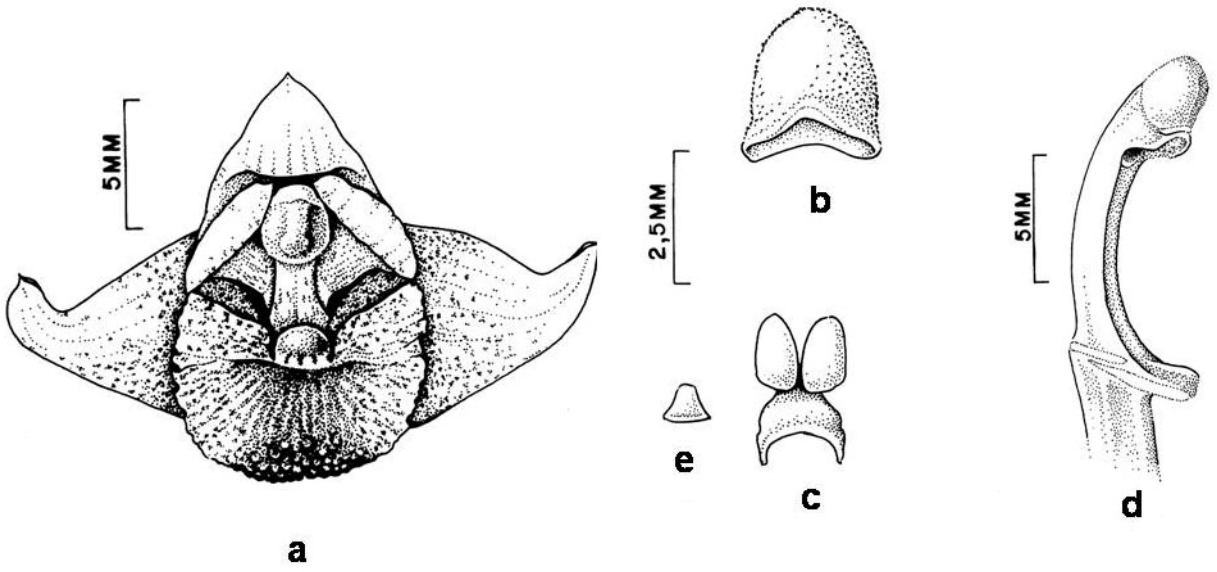


Fig. 2. *Maxillaria subulata* var. *madida*. (a) Flower. (b) Anther cap. (c) Pollinarium (d) Column, lateral view. *Maxillaria vernicosa.* (e) Pollinarium without pollinia.

The callus in this group comprises a fleshy longitudinal and glabrous ridge extending from the lip base up to its median region along the midvein, varying from oblong to clavate. As in the midlobe region of the lip, the callus may also have several conical to viliform papillae (Davies & Turner 2004).

The column is semi-cylindrical, elongated, slightly bent downwards, and may be glabrous or present conical and/or obpyriform papilae (Davies & Turner 2004) (Fig. 2D). Its base bears a ventral extension, the column foot, to which the lip is attached and articulated (Fig. 2D). The anther is hood-shaped, terminal, incumbent, 4-celled, and externally glabrous or papillose (Figs. 1E and 2B; Davies & Turner 2004). It bears two paired laterally flattened, superposed, ovate to round pollinia (Fig. 2C). The pollinarium also has a small tegular stipe, extremely long in *M. pacholskii* and *M. uncata*, and an arcuate viscidium (Fig. 2C), the exception being *M. vernicosa*, which has a truncate viscidium (Fig.

2E). The stigma is concave. The ovary is ridged, erect, tricarpelar and unilocular. The pedicel is well differentiated from the ovary and commonly concealed by scape bracts.

The capsules are always erect and green when developing with perianth segments persistent to dehiscent. When the fruits are ripe, the six valves split open from the apex (never separate widely in *M. nardoides*). Fruits usually take 3-5 months to mature. Hygroscopic trichomes (elaters) are commonly found within the fruits of species within this group, but in *M. nardoides* the aggregation of such trichomes is quite unique, reaching up to 5.5 cm (Blanco et al. 2006). We examined seeds from *M. acicularis, M. neowiedii* (Fig. 1F) and *M. vernicosa* under the SEM (vouchers deposited at SP). They correspond to the '*Maxillaria*' type (Dressler 1993) in which they are dust-like (varying from 210 to 516 µm long), dark yellow to brownish, bottle-like in form, and have isodiametric (or nearly so) terminal testa cells and medial sector cells rectangulate and strongly elongate, with cell-borders smooth and regularly thickened. (Fig. 1F; see also Blanco et al. 2006).

Taxonomic Treatment

Epiphytic or rupicolous herbs, caespitose or rhizomatous, from 2 - 30 cm tall. Roots cylindrical, white, with a well developed velamen and commonly with annular thickenings. Rhizome aerial, short to elongate, covered by papery dry bracts, with pseudobulbs. Pseudobulbs 8.5 – 48 x 1 – 23.5 mm, conical to cylindrical, rarely lenticular and flattened, smooth to ridged, light to dark green, apically 1-2 (3-4)-foliate, always terminating the shoot. Leaves 11 - 235 x 0.4 – 14 mm, up to 4 mm thick, sessile, erect or twisted, flat or conduplicate, coriaceous, fleshy or, more rarely, membranaceous, lanceolate. Inflorescences, 1-many, generally 2 in each pseudobulb, reduced to a 1-flowered peduncle per bract (rhizome scale) at the pseudobulb base; pedicel present and well-diferentiated from ovary, pedicel and ovary length 11.5 – 36.5 mm. Flowers 6 – 21 mm in length, resupinate, campanulate to spreading, mostly not fragrant, when so sweetly or fruity like; colouration varying from white, yellow, red, lavender to purple or tan to brownish. Perianth segments elliptic, oblong or obovate, rounded and apiculate at the apex and truncate at the base. Dorsal sepal 5.5 – 23 x 2 – 8.5 mm, the lateral ones 6 – 22 x 1.5 – 10.5 mm, also ovate to triangular. Spur inconspicuous. Petals 5 – 20 x 1 – 9 mm.

Labellum 5.5 – 21 x 3 – 19 mm, varying from lanceolate to obovate, the apex attenuate to cuneate, entire to slightly 3-lobed, commonly with a conspicuous dark-red spot at the midlobe lip. Callus oblong or clavate, up to the midportion of the lip. Column 2,5 – 13.5 mm. Pollinarium 2 – 10 mm in length, usually bearing a very small tegular stipe, 0.8 – 3 mm long, and an arcuate viscidium up to 2 mm wide. Fruits 10 – 300 mm in length, commonly bearing hygroscopic trichomes among seeds.

Key to taxa belonging to the *'Maxillaria madida'* complex

1. Pseudobulbs bearing one leaf

2. Pseudobulbs flattened …….. *M. ferdinandiana*

2'. Pseudobulbs conical

3. Perianth segments linear; pollinarium with a long stipe, 2 – 3 mm long; plants occurring in northwestern South America and Central America

4. Leaves always linear, acute; flowers dark purple; foot about half as long as column ……... *M. pacholskii*

4'. Leaves very variable, generally elliptic to oblong, sometimes linear; flowers waxy white to light purple, commonly with pinkish stripes; foot almost as long as column …..... *M. uncata*

3'. Perianth segments oblong; pollinarium with a short stipe, 1 mm long; plants occurring in southeastern South America, mainly in Brazil

5. Plants up to 5.5 cm tall; leaves 18 - 39 mm in length; lip length 6.5 – 9.5 mm …….. *M. pumila*

5'. Plants up to 30 cm tall; leaves 36 – 235 mm in length; lip length 10 – 21 mm

6. Pseudobulbs commonly ridged when mature; leaves cylindrical; flowers reddish to brownish; callus oblong …… *M. subulata* var. *monophylla*

6'. Pseudobulbs commonly smooth when mature; leaves lanceolate and extremely fleshy; flowers pale yellow, rarely pale red; callus clavate ……... *M. cepula*

1'. Pseudobulbs bearing more than one leaf

7. Pseudobulbs bearing 3-4 aciculate leaves …… *M. nardoides*

7′. Pseudobulbs bearing two aciculate, cylindrical or lanceolate leaves

8. Leaves acicular

9. Flowers up to 10 mm long, whitish to pinkish or yellowish, never with dots

10. Flowers vivid yellow; lateral sepals ovate to triangular; petals oblanceolate; lip oblong, with a conspicuous red spot in the posterior region and a rounded and retuse apex; callus claviform or retuse, 3-5 mm long ……. *M. vernicosa*

10′. Flowers pinkish to white; sepals and petals elongate, conspicuously ligulate to lanceolate; the lip lanceolate, white, sometimes with pink or pale yellow stripes and an acute apex; callus inconspicuous, 3 mm long ……..…… *M. echiniphyta*

9′. Flowers 11 – 40 mm long, red-brownish to chocolate purple, very rarely pale yellow, sometimes with red dots

10. Petals elliptic to lanceolate; leaves rarely longer than 50 mm; ovary plus pedicel generally longer (or same height) than adjacent pseudobulb ….……... *M. neowiedii*

10′. Petals oblanceolate to oblong; leaves commonly longer than 50 mm; ovary plus pedicel always shorter than adjacent pseudobulb …… *M. subulata* var. *acicularis*

8′. Leaves cylindrical or lanceolate

11. Flowers not fragrant; ovary-pedicel generally longer (or same height) than the adjacent pseudobulb; leaves rarely longer than 50 mm …….....……. *M. neowiedii*

11′. Flowers with a fruity fragrance; ovary-pedicel always shorter than the adjacent pseudobulb; leaves usually greater than 50 mm

12. Leaves cylindrical (strongly conduplicate) and fleshy; plants usually found as rupicolous in rock outcrop formations of Minas Gerais .....
…… *M. subulata* var. *subulata*

12′. Leaves lanceolate and membranaceous; plants found as epiphytes in seasonally dry and humid Atlantic forests in south and southeastern Brazil …….
……...……...……...…… *M. subulata* var. *madida*

1. *Maxillaria cepula* Rchb.f., Bonplandia 3: 216 (1855). ≡ *Maxillaria madida* var. *cepula* (Rchb.f.) Hoehne in Arq. Bot. Estado São Paulo 2: 136 (1952). Type: Brazil, Rio de Janeiro, *s. col., s.d.* (holotype, not found).

= *Maxillaria pachyphylla* Schltr. ex Hoehne, Bol. Mus. Nac. Rio de Janeiro, Bot. 12(2): 36 (1936) **syn. nov.**

= *Maxillaria pachyphylla* var. *brunneofusca* Hoehne, Bol. Mus. Nac. Rio de Janeiro, Bot. 12(2): 36 (1936) **syn. nov.**

= *Maxillaria cepula* var. *pallida* Klinge, Acta Horti. Petrop. 17, fasc. 1 (2): 10 (1898). ≡ *Maxillaria madida* var. *pallida* (Klinge) Cogn., Fl. bras. (Mart.) 3(6): 70 (1904) **syn. nov.**

Figs. 3A-D and 9A

Additional illustrations: Saund. Ref. Bot. 2, tab. 104 (1872); Acta Hort. Petropol. 17(1,2): tab. 2, fig. 17 (1898); Fl. bras. (Hoehne) 10 (12, 7), tab. 151(3), 152, 153(2) (1953).

Description: Epiphytes, rarely rupicolous plants, caespitose, 7 - 16 cm tall. Pseudobulbs conical-cylindrical, commonly smooth when mature, dark green, 13 – 46 x 3 – 9 mm, bearing one leaf. Leaves lanceolate, flat to slightly conduplicate, very fleshy, 55 – 112 x 6 – 14 mm, 1 – 3.5 mm thick. Flowers pale yellow with red dots, rarely reddish, dark yellow when old, fragrance reminding of baby powder, 13 – 20 mm long. Perianth segments never revolute. Dorsal sepal oblong, apiculate, 14 – 20 x 4.5 – 7.5 mm; lateral sepals triangular, apiculate, 16 – 22 x 6.5 – 10.5 mm. Petals oblong, 11 – 18 x 3.5 – 6.5 mm, apex rounded. Lip oblanceolate, 14 – 21 mm when expanded; midlobe apex rounded and commonly retuse; callus 7.5 – 1.5 mm long, clavate. Column 7 – 12.5 mm long; foot 4.5 – 10 mm long. Anther cap 3.5 – 5.5 x 2.5 – 5 mm; pollinia 1.5 – 3 x 1.5 – 2 mm; viscidium arched, 2 – 4.5 mm long, stipe 1 – 2.5 mm long. Ovary and pedicel 17.5 – 32 mm long, always shorter than the adjacent pseudobulb.

Distribution and ecology: States of Espírito Santo, Rio de Janeiro, São Paulo and Paraná in Brazil, mainly in coastal regions. Seasonally dry to wet Atlantic forest, 0 – 1000 m.

Diagnostic characters: This species can be easily identified by its smooth, conical-cylindrical pseudobulbs bearing one rather thick lanceolate leaf and by its pale yellow flowers.

Comments: The name *Maxillaria pachyphylla* has been widely used for identification of this species, especially after the revision of Hoehne (1953), who placed *M. cepula* under synonym with *M. madida*. Although the holotype of *M. cepula* could not be located, a careful examination of its protologue indicated it comprises a good species, under which *M. pachyphylla* should be placed as a synonym. Although this is one of the least variable species in this group, the difference in flower colouration (pale yellow x reddish) was considered by Hoehne (1936) a good character for the description of different varieties. However, the analysis of herbarium material and cultivated specimens indicated the occurrence of reddish flowers to be extremely rare and restricted to a few individuals. Therefore, we here do not consider the varieties proposed by Klinge (1898) and Hoehne (1936) as good taxa.

Specimens examined[9]*:* **BRAZIL. Espírito Santo:** intersection among Domingos Martins - Alfredo Chaves - Guarapari, 14.iv.1972, *Kautsky 363* (HB). **Paraná:** Cerro Azul, Morro Grande, ix.1963, *Hatschbach 3241* (HB); Telêmaco Borba, Fazenda Monte Alegre, (fl cult) 9.ix.2003 (ESA). **Rio de Janeiro:** Rio de Janeiro, estrada da Guanabara, mata da Tijuca, 25.xi.1969, *Sucre et al. 6363* (RB). **Santa Catarina:** 20.x.1940, *Brade sn* (RB); Santa Rita, rio São Lourencinho, vii.1961, (fl cult) 16.i.2003 (SP). **São Paulo:** Atibaia (UEC); Barra do Turvo, road BR-116, 8.ii.1995, *Leitão Filho et al. 32875* (UEC); Capão Bonito, Fazenda Boa Esperança, 25.viii.2003, (fl cult) 9.ix.2003 (ESA); Jundiaí, Serra do Japi, 13.xii.2002, *Pansarin 76* (UEC); Juqui (Juquitiba), zona litorânea, (fl cult) 11.xi.2002, *Pires sn* (SP); Maresia, Bertioga - São Sebastião road, (fl cult) 9.ix.2003 (ESA); Registro, rio Ribeira, (fl cult) 9.ix.2003 (ESA); São Paulo, São Paulo, Instituto de Botânica, viii.1976, (fl cult) 8.ix.2003, *Bicalho sn* (SP); São Sebastião, Juréia, 20-21.ix.1984, (fl cult) 22.xi.2001, *Gil et al. sn* (SP); Teodoro Sampaio, Morro do Diabo, (fl cult) 11.xi.2002, *s. col.* (SP)

2. *Maxillaria echiniphyta* Barb. Rodr., Gen. Sp. Orchid. 1: 122 (1877)

Type: Brazil, Rio de Janeiro, Serra do Mar, *J. Barbosa Rodrigues, s.d.* (holotype, missing). Lectotype, here designated: Barbosa Rodrigues J., Iconographie des Orchidées du Brésil, tab. 280, vol. 6, reproduced by Sprunger et al. (1996)..

[9] O número de herbário dos espécimes coletados durante o desenvolvimento desse estudo não estavam disponíveis na elaboração desta tese.

= *Maxillaria seidelii* Pabst, Orquídea 22: 52 (1960) **syn. nov.**

Figs. 3E-G and 9B

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 14(2); Fl. bras. (Hoehne) 10 (12, 7), tab. 159(3) (1953), Butzin & Senghas, Schltr. Die Orchideen, fig. 1669 (1996)

Description: Epiphytes up to 5 cm tall, caespitose. Pseudobulbs conical, smooth to ridged, light green, 9 – 17 x 1 – 2 mm, bearing two leaves. Leaves aciculate, cylindrical, 20 – 30 x 0.5 – 1 mm. Flowers pinkish to white, never with dots, generally not fragrant, 8 – 10 mm long. Flower segments revolute or not. Sepals and petals ligulate to lanceolate with an acute apex; dorsal sepal 8 – 10 x 2 mm; lateral sepals 8 – 10 x 1.5 – 2 mm; petals 6 – 8 x 1 – 1.5 mm. Lip lanceolate, 7 – 10 X 2 mm when expanded, white, sometimes with pink or pale yellow stripes, lateral lobes slightly erect, inconspicuous; midlobe apex acute; callus 3 mm long, inconspicuous. Column 3 – 4 mm long; foot 2 – 3 mm long. Anther cap 1 x 1 mm; pollinia 1 x 1 mm; viscidium arched, 1 mm long, stipe 1 mm long. Ovary and pedicel 18-20 mm long, always longer than adjacent pseudobulb.

Distribution and ecology: Restricted to the coastal Atlantic Forest in the state of Rio de Janeiro, Brazil. Rare and severely threatened. It is still found for sale by orchid growers in southeastern Brazil.

Diagnostic characters: Together with *Maxillaria pumila* and *M. vernicosa, M. echiniphyta* is one of the smallest species of this group and the only one from southeastern Brazil with white to pale pink flowers and ligulate to lanceolate perianth segments.

Comments: The examination of the holotype of *M. seidelii* revealed that this species is very similar to *M. echiniphyta* concerning flower morphology and vegetative traits. For this reason we propose the reduction to *M. seidelii* to synonymy under *M. echiniphyta.* Considering the absence of an holotype and the rarity of this species, a lectotype for *M. echiniphyta* is here proposed based on the illustration cited in the protologue.

Specimens examined: **BRAZIL.** *s.loc., Koehler 111a,* 25.viii.2002 (UEC). **Rio de Janeiro:** Petrópolis, Correas, 8.xi.1927, *Wiltusnig & Spannagel 107* (R); Petrópolis, xi.1934, *Spannagel sn* (RB); Rio de Janeiro, Paineiras, 16.iv.1923, *Ochionni sn* (RB); Rio de Janeiro, Cascadura, Serra da Bica, 18.vii.1890, *Schwacke 6981* (RB)

3. *Maxillaria ferdinandiana* Barb. Rodr., Gen. Sp. Orchid 2: 204 (1881)

Type: Brazil, [Rio de Janeiro, Paulo de Frontin], Rodeio; Minas Gerais, Juiz de Fora, *J. Barbosa Rodrigues, s.d.* (holotype, missing). Lectotype, here designated: Barbosa Rodrigues J., Iconographie des Orchidées du Brésil, tab. 288A, vol. 6, reproduced by Sprunger et al. (1996).

Figs. 3H-I and 9C

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 16(1); Fl. bras. (Hoehne) 10 (12, 7), tab. 155 (1953)

Description: Epiphytes, 5 – 10 cm tall, caespitose. Pseudobulbs lenticular, flattened and commonly smooth, yellowish to light green, 17 – 23 x 7 – 9.5 mm, bearing one leaf. Leaves lanceolate, membranaceous, flat, 27 – 65 x 5 – 10 mm. Flowers yellowish, rarely reddish, never with dots, not fragrant, 12 – 14 mm long. Flowers segments never revolute. Dorsal sepal oblong to obovate, 11 – 14 x 4.5 – 8 mm; lateral sepals triangular to ovate, apiculate, 12 – 14.5 x 5 – 9.5 mm. Petals oblanceolate to oblong, 10.5 – 11.5 x 3.5 – 5.5 mm, apex rounded. Lip obovate, 10 – 13 x 6.5 – 8.5 mm when expanded; midlobe apex rounded to retuse; callus 5 – 6.5 mm long, clavate, with an prominent apex. Column 7 – 9 mm; foot 3.5 – 6 mm long. Anther 1.5 – 3.5 x 2 – 3.5 mm; pollinia 1.5 – 2 x 1 – 1.5 mm; viscidium arched, 1 – 2.5 mm long; stipe 1 – 2 mm. Ovary and pedicel 15 – 25 mm long, always shorter than adjacent pseudobulb.

Distribution and ecology: South to southeastern Brazil, from Rio Grande do Sul to Minas Gerais. Seasonally dry to wet Atlantic forest, 100 – 800 m.

Diagnostic characters: Although variation on sequence data presented by S. Koehler et al. (unpubl. data, Cap. 1) was not informative enough to distinguish *M. ferdinandiana* from the polymorphic '*M. neowiedii*' complex, this species presents very clear morphological diagnostic characters and can be easily distinguished from the others within this group by its flattened and commonly smooth unifoliate pseudobulbs, and by its yellow to pale green flowers, always shorter than the adjacent pseudobulb.

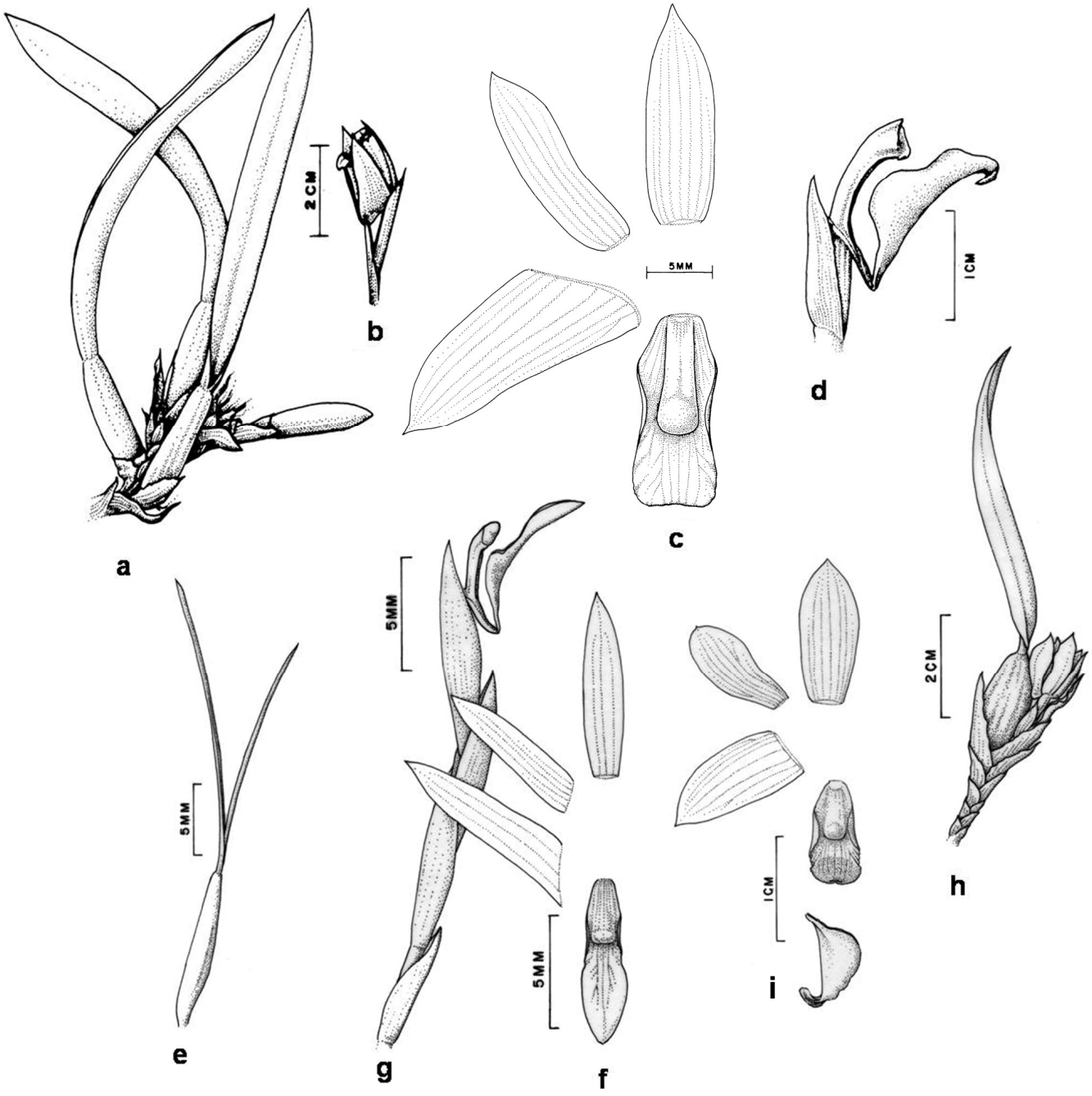


Fig. 3. *Maxillaria cepula* [*Gil et al. sn,* SP]. (a) Habit. (b) Flower, lateral view. (c) Flower segments. (d) Column, lateral view with lip attached. *Maxillaria echiniphyta* [*Koehler 111a,* UEC]. (e) Habit. (f) Flower segments. (g) Column, lateral view with lip attached. *Maxillaria ferdinandiana* [*Koehler 165,* UEC]. (h) Habit. (i) Flower segments.

Comments: Since the holotype of *M. ferdinandiana* deposited could not be located (possibly never existed), a lectotype is here proposed based on the illustration present in the protologue.

Specimens examined: **BRAZIL.** *s. loc., Koehler 165,* 25.viii.2002 (UEC). **Espírito Santo:** Rio Doce, matas do rio São Gabriel, ix.1950, *Vieira sn* (RB); Vitor Hugo, 5.x.1978, *Seidel 1219* (HB). **Minas Gerais:** Camanducaia, Fazenda Levantina e Vila Monte Verde, (fl cult) 1.xi.2001, 27.ix.2002 (SP); Carangola, rio Carangola, mata ciliar, x.1985, *Leoni 30* (HB); Juiz de Fora, Fazenda São Mateus, matas do rio do Peixe, 20.vii.1968, *Vasco Gomes 16* (HB); Poços de Caldas, xii.1981, (fl cult) xii.2002, *Barros sn* (SP); Rio Novo, *Schwacke 11123* (RB); **Paraná:** Curitiba, 1944, (fl) 10.ix.1945, *Guimarães sn* (RB). **Rio Grande do Sul:** Herval, matas da cascata, xii.1969, *Fersch 133* (HB); Nova Hamburgo, norte, em beira de rodovia, xi.1960, *Birck sn* (HB). **Rio de Janeiro:** Areal, ilha do rio Paraibuna, 16.x.1949, *Pabst 421* (HB); Nova Friburgo, ix.1954. *Pinto de Lima 183* (HB); Petrópolis, Versim, *Spannagel 106* (HB); Teresópolis, 26.ix.1958, *Abendroth p-92 616* (HB). **Santa Catarina:** Campo Alegre, 4.x.1969, *Seidel 986* (HB); Ibirama, 25.x.1950, *Gevieski 1* (HB); Ilha de Santa Catarina, rio Vermelho, 18.x.1953, *Rohr 2249* (HB); Orleans, arredores, Propriedade Rio Novo, vii.1962, (fl cult) 20.ii.2003, 27.ix.2003, *Bicalho & Targa sn* (SP); Sombrio, Pirão Frio, 5.ix.1958, *Reitz & Klein 9058* (HB). **São Paulo:** Amparo, Três Pontes, vi.1926, *Sampaio 4540* (RB)

4. *Maxillaria nardoides* Kraenzl., Repert. Spec. Nov. Regni Veg. 1: 90 (1905) [10]

Type: Peru, Depto Junin, Prov. Tarma, 1000 m, *Weberbauer 1422, s.d.* (holotype, destroyed).

= *Maxillaria juergensii* sensu Vasquez & Dodson, Icon. Pl. Trop., ser 1, 6: 554 (1982), non Schlechter (1925)

Figs. 4 and 11

Additional illustrations: Ic. Pl. Tropicarum, 554 (1982); Ic. Orchidacearum Peruvianum, 107 (1993); Orchids (Lindleyana), 686-690 (2006)

[10] Considerando que esta é uma espécie rara e extra-Brasileira, raramente com material fértil coletado, sua descrição foi complementada com informações de Vásquez & Dodson (1982), Bennett & Christenson (1993) e Blanco *et al.* (2006).

Description: Epiphytes, caespitose or pendent, 3 – 5 cm tall. Pseudobulbs cylindrical, ridged, dark green, 10 – 25 x 4 – 5 mm, 3 – 4 leaves each. Leaves acicular, conduplicate, 0.7 – 25 x 7 – 10 mm. Perianth segments vary from cream suffused and spotted with red to uniformly red purple, revolute and denticulate, 10 – 15 mm long. Dorsal sepal oblong-elliptic, apiculate, 9 – 11 x 3 - 5 mm, lateral sepals similar, 10 – 11 x 3.5 – 6 mm. Petals elliptic to oblanceolate, 9 – 11 x 3 -5 mm, apiculate. Lip elliptic to oblong, 10 – 13 x 5 – 7 mm when expanded, slightly lobulate; midlobe recurved, shallowly retuse; callus 4 – 5 mm long, oblong. Column 9 - 12 mm long. Anther 0.5 x 0.5 mm; viscidium arched, 1 mm long; stipe 2 mm; pollinia 2 x 2 mm. Ovary and pedicel longer than adjacent pseudobulb. Fruits 10 – 12 mm long, very particular in this species for extruding a mass of hygroscopic trichomes when mature, which varies from 35 – 65 mm long. Seeds are interspersed among trichomes

Distribution and ecology: Bolivia, Colombia, Ecuador and Peru. Montane rain forest, 300 – 1200 m along the eastern slope of the Andes.

Diagnostic characters: This species can be easily identified by its pseudobulbs bearing 3-4 acicular leaves, which are unique in this group.

Comments: The holotype of *M. nardoides* could not be located – it was probably destroyed at B) (Butzin 1981).

Specimens examined: **COLOMBIA**. **Caquetá:** comissaria, *O. Renz s.n.,* iv. 1938 (RENZ). **BOLIVIA. Cochabamba:** Prov. Carrasco, Chipiriri, *Vásquez 52* (SEL). **ECUADOR. Morona-Santiago:** Carretera Bomboiza-Gualaquiza, 3º27´S 78º34´W,2-3.xi.1986, *Zaruma 356* (MO). **Pastaza:** Pastaza Cantón, 1º34' S 77º25' W, 4-21.x.1990, *Gudiño et al. 966* (MO, NY). **PERU. San Martin:** Moyobamba, 4 km downstream río Mayo, 1 km NE from bank, 18.vi.1977, *Bennett & Lopez 3212* (Bennett & Christenson 2002); Tarapoto, along road to Yurimaguas, 2.xii.1987, *Bennett 4167* (USM). **Pasco:** Oxapampa, Puerto Bermudez, 30.viii.1990, *del Castillo ex Bennett 4692* (USM)

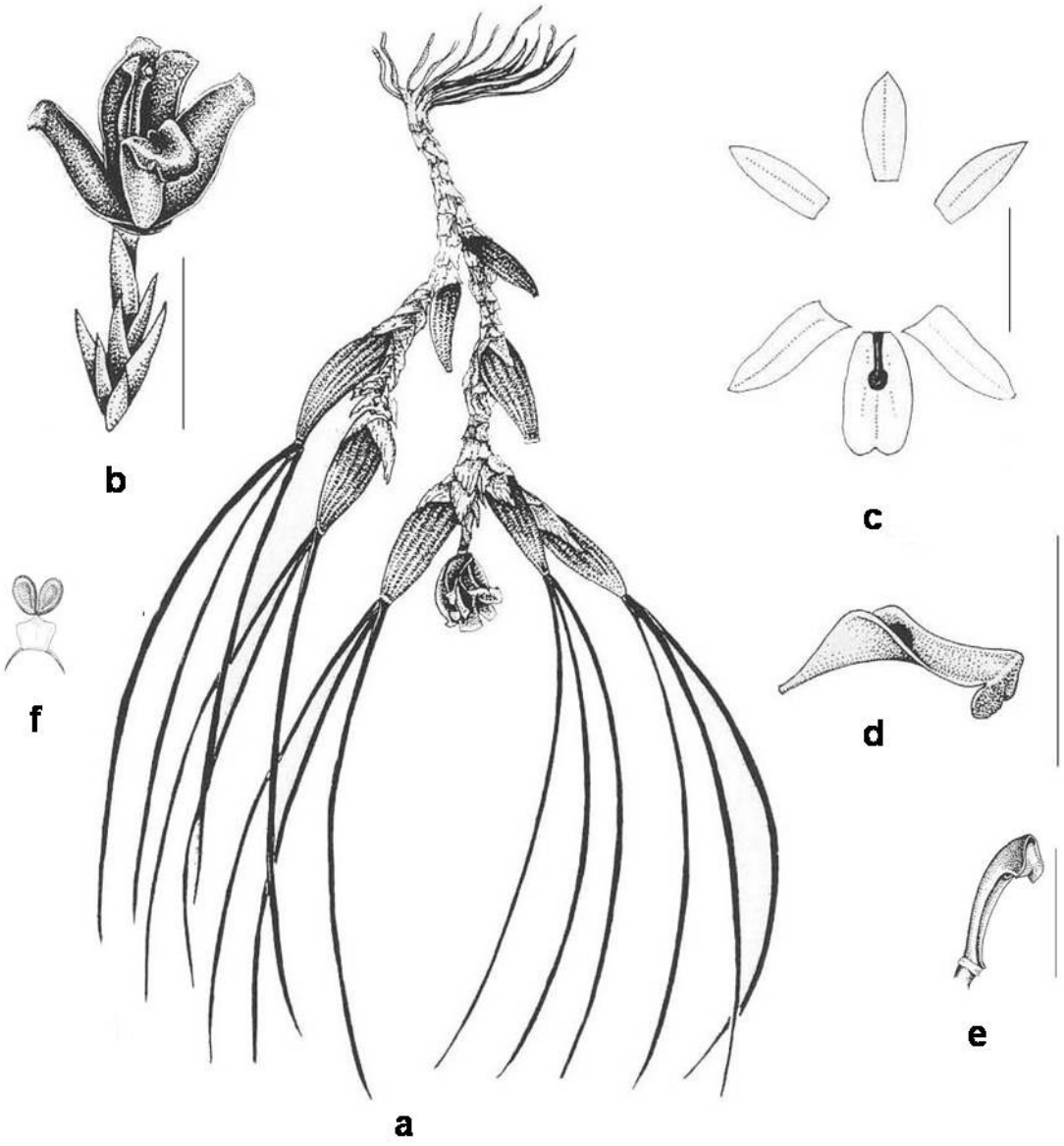


Fig. 4. *Maxillaria nardoides*. (a) Habit. (b) Flower. (c) Flower segments. (d) Lip, lateral view. (e) Column. (f) Pollinarium. (Scale bar 1 cm; modified from Vasquez & Dodson, 1982).

5. *Maxillaria neowiedii* Rchb. f., Linnaea 41: 29 (1877)

Type: Brazil, *s.col., s.d.* (holotype, not found).

= *Maxillaria paranaensis* Barb. Rodr., Gen. Sp. Orchid. Nov. 2: 205 (1881) **syn. nov.**

= *Maxillaria spegazziniana* Kraenzl., Orchis 2: 51 (1908) **syn. nov.**

= *Maxillaria juergensii* Schltr., Repert. Spec. Nov. Regni Veg. Beih. 35: 88-89 (1925). **syn. nov.**

= *Maxillaria acicularis* var. *brevifolia* Cogn., Fl. bras. (Mart.) 3: 72 (1904) **syn. nov.**

= *Maxillaria cogniauxiana* Hoehne, Bol. Agric. (São Paulo) 34: 632 (1933) **syn. nov.**

= *Maxillaria cogniauxiana* var. *longifolia* Hoehne, Bol. Agric. (São Paulo) 34: 633 (1933) **syn. nov.**

= *Maxillaria heterophylla* var. *acicularifolia* Hoehne, Arq. Bot. Estado São Paulo n.s. 2: 133 (1952) **syn. nov.**

= *Maxillaria heterophylla* var. *longifolia* Hoehne, Arq. Bot. Estado São Paulo n.s. 2: 133-134 (1952) **syn. nov.**

= *Maxillaria heterophylla* var. *intermedia* Hoehne, Arq. Bot. Estado São Paulo n.s. 2: 132 (1952) **syn. nov.**

= *Maxillaria heterophylla* var. *magnifolia* Hoehne, Arq. Bot. Estado São Paulo n.s. 2: 134 (1952) **syn. nov.**

= *Maxillaria heterophylla* var. *latifolia* Hoehne in Arq. Bot. Estado São Paulo n.s. 2: 133 (1952) **syn. nov.**

= *Maxillaria heterophylla* var. *pygmaea* Hoehne in Arq. Bot. Estado São Paulo n.s. 2: 134 (1952) **syn. nov.**

Figs. 5 and 9D

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 17(1), 21(2); Fl. bras. (Hoehne) 10 (12, 7), tab. 143(3), 150(1, 4), 151(2), 157(3), 159(1) (1953); Las Orquídeas del Parque Nacional Iguazú, 125 (2001).

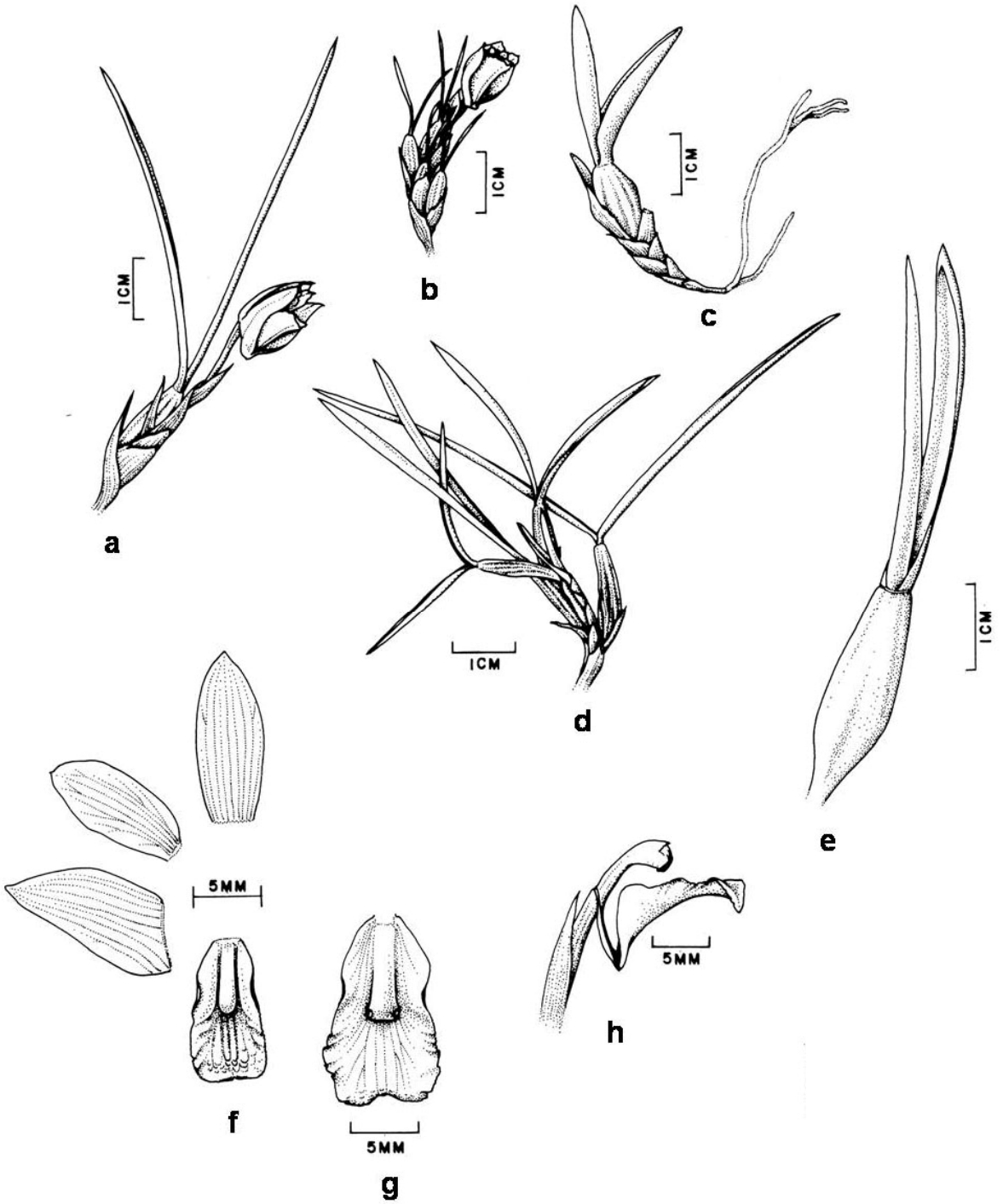


Fig. 5. *Maxillaria neowiedii*. (a-e) Habit, showing variation in leaf morphology. (f) Flower segments. (g) Detail of lip, showing variation on size. (h) Column, lateral view with lip attached.

Description: Epiphyte or rupicolous plants, caespitose or pendent, 2.5 – 10 cm tall. Pseudobulbs conical, smooth to ridged when mature, light to dark green or brownish, 16 – 24.5 x 3 – 7 mm, bearing two leaves. Leaves aciculate to cylindric, conduplicate, or lanceolate and flat, membranaceous to coriaceous, (11-) 30-50 (-80) x 0.5 – 8 mm, 0.5 – 2.5 mm thick. Flowers cream to tan-colored or reddish to chocolate purple, usually with red dots, not fragrant, 11 – 40 mm long. Flower segments sometimes revolute. Dorsal sepal oblong to ovate, 8 – 18 x 4 – 7 mm; lateral sepals ovate to triangular, apiculate, 7 – 19 x 4.5 – 10 mm. Petals elliptic to oblong-lanceolate, 8 – 17 x 2 – 7.5 mm, apex rounded. Lip obovate to oblanceolate, curved downwards or straight, 7 – 17 x 4 – 10.5 mm when expanded; midlobe orbicular; apex rounded to retuse, the callus 4 – 9 mm long, generally clavate, rarely oblong. Column 5.5 – 11.5 mm; foot 1.4 – 8 mm long. Anther 1.5 – 4 x 1 – 4 mm; pollinia 1 – 2 x 2 – 2.5 mm; viscidium arched, 1 – 3 mm long; stipe sometimes almost absent, 0.1 – 2 mm. Ovary and pedicel 11.5 – 36.5 mm long, generally longer than adjacent pseudobulb

Distribution and ecology: South to southeastern Brazil, from Rio Grande do Sul to Minas Gerais states. Seasonally dry to wet forest formations, 300 – 1800 m.

Diagnostic characters: Bifoliate, cylindrical pseudobulbs, generally reddish to dark purple flowers, with ovary and pedicel length as long as or longer than the adjacent pseudobulb.

Comments: M. neowiedii is one of the most variable species of this group, considering the shape and size of leaves and flower size and colour. The analysis of herbarium specimens as well as cultivated plants from many different localities showed that the different species and varieties suggested for this complex (see review in Hoehne 1953) belong to a continuous range of morphological variation. A phylogenetic study for the '*Maxillaria madida*' complex developed by Koehler et al. (unpubl. data; Cap. 1) indicated *M. neowiedii*, with all its infra-specific variation, comprises a monophyletic group together with *M. ferdinandiana*. Since there are good diagnostic morphological characters to distinguish the '*M. neowiedii*' group from *M. ferdinandiana* (see above), we propose the recognition of this complex as a single species, *M. neowiedii*.

Contrary to *M. subulata*, infra-specific delimitation within *M. neowiedii* remains unclear, since morphological variants are not as clearly defined as in *M. subulata* and there

is no correlation between geographical or ecological occurrence with morphological variation. In fact it is possible to observe in the same locality individuals with flat, lanceolate leaves and reddish, smaller flowers blooming together with needle–like leaved specimens with larger, dark purple flowers, having intermediate morphotypes growing between them (S. Koehler, pers. obs.). Considering our present lack of knowledge on phylogenetic patterns within this group, we suggest a conservative approach, recognizing a single, *M. neowiedii*, defined by the diagnostic morphological characters above described.

The holotype of *M. neowiedii* could not be located at HBG nor W, where most of Reichenbach´s types were deposited. References of the type material and duplicates were also checked at K and AMES, but were not found.

Specimens examined: **BRAZIL. Espírito Santo:** Castelo, 19.vii.1975, *Seidel 1140* (HB); Domingos Martins, Aracê, 7.ii.1973, *Hatschbach & Ahumada 31363* (HB); Santa Leopoldina, xi.1974, *Seidel 1116* (HB); Santa Teresa, 5.x.1969, *Seidel 987* (HB). **Minas Gerais:** Alagoa, 12.v.2002, *Faria & Ribeiro sn* (UEC); Alto de Caparaó, Serra do Caparaó, ix.1941, *Brade 17078* (HB); Camanducaia, Vila Monte Verde, Fazenda Levantina, (fl cult) 27.ix.2002 (SP). **Paraná:** Bituruna, Salto Grande, rio Iguaçu, 17.x.1966, *Hatschbach 14959* (HB); Campina Grande do Sul, Serra Capivari Grande, 15.i.1969, *Hatschbach & Koczick 201781* (HB); Curitiba, Paranaguá road, (fl) 9.ix.2003 (ESA); Francisco Beltrão, 28.vii, 1970, *Leinig 452* (HB); Ipiranga, Coatis, 8.x.1969, *Hatschbach 22377* (HB); Jaguaritiba, 68 km from Curitiba, 12.xi.1961, *Pabst 6733 Pereira 6907* (HB); Mandirituba, 10.xi.1972, *Dombrowski 4279 & Kuniyoshi 3460* (HB); Morretes, Pilão de Pedra, 18.iii.1966, *Hatschbach 14069* (HB); Palhoça, São Bonifácio, 15.xi.1954, *Rohr 2253* (HB); Piraí do Sul, xi.1972, *Seidel 1056* (HB); Piraquara, Borda do Campo, 28.x.1969, *Leinig 417* (HB); Porto Vitória, 16.x.1966, *Hatschbach 14918* (HB); Quatro Barras, 28.x.1969, *Leinig 409* (HB, K); Rio Negro, 15.x.1953, *Hatschbach 3238* (HB); São João do Triunfo, 7.xi.1967, *Hatschbach 17729* (HB); São José dos Pinhais, Saltinho, 21.ix.1952, *Hatschbach sn* (HB); Sapopema, Salto das Orquídeas, 27.ix.1997, *Medri et al. 449* (UEC); Ventania, 16.x.1972, *Seidel 1047* (HB); Vossoroca, 20.ii.1955, *Welter 127* (HB). **Rio Grande do Sul:** Canela, viii.1950, x.1950, *Richter sn* (HB); Montenegro, Estação São Salvador, vii.1951 (HB); Santa Maria, 21.ix.2002, *Breier sn* (UEC); São Francisco de Paula, 18.xii.1950, *Bambo HA-49445* (HB). **Rio de Janeiro:** Itatiaia, 14.xi.1952, *Pabst sn* (HB); Nova Friburgo, Lumiar, (fl cult) 11.xi.2002 (SP); Nova Friburgo, Macaé de Cima, xii.2003, *Koehler & Pinheiro sn* (UEC); Petrópolis, *Spannagel sn* (HB); Resende, Mauá, xi.1952, *Welter sn* (HB); Rio de Janeiro, morro do Archer, x.1928, Brade 8560 (HB); Serra dos Órgãos, 16iv.1972, *Emmerich 3675* (HB); Teresópolis, Parque Nacional da Serra dos Órgãos, (fl cult) 12.iii.2004 (ESA). **Santa**

Catarina: Blumenau-Lajes road, Braço do Trombado, 3.viii.1967, *de Haas 5866* (HB); Biguaçú, 21.vii.1951, *Reitz 4128* (HB); Caçador, rio dos Bugres, 13.vii.1962, *Reitz & Klein 13163* (HB); Campo Alegre, 19.vii.1975, *Seidel 1141* (HB); Canoinhas, rio dos Pardos, 15.ix.1962, *Klein 3024* (HB); Corupá, 30.vi.1959, *Welter sn 243* (HB); Nova Teutonia, 25.v.1944, *Plaumann 446* (RB); Orleans, Propriedade Rio Novo, vii.1962, (fl cult) 27.ix.2002, *Bicalho & Targa sn* (SP); Palhoça, morro do Cambirela, 26.ix.1957, *Rohr* 2322 (HB); Papanduva, Serra do Espigão, 3.i.1962, *Reitz & Klein 11401* (HB); Porto União, 16.ix.1962, *Klein 3074* (HB); Rio do Sul, Serra do Matador, 1.viii.1958, *Reitz & Klein 6877* (HB); São Bento, 27.vi.1885, *Schwacke 5508* (RB); São José, Serra da Boa Vista, 14.x.1960, *Reitz & Klein 10202* (K); Sertão da Lagoa, 31.xii.1950, *Rohr 2050* (HB); Tijucas, Pinheiral, 12.i.1953, *Rohr 2070* (HB); Urussanga, Pinhal da Companhia Lauro Muller, 23.viii.1958, *Reitz & Klein 7056* (HB). **São Paulo:** Apiaí, Banhado Grande, ix.1971, (fl cult) 9.ix.2003 (ESA); Bananal, Bocaína, x.1949, *Brade & Duarte 20151* (RB); Campos do Jordão, 22.xii.1997, *E.R. Pansarin et al. 196* (UEC); Cotia, Reserva Florestal Morro Grande, v.1998, (fl cult) 27.ix.2002, *Catharino et al. sn* (SP); Itaperaí, Fazenda São Francisco, *Brolio sn,* vii.1976, (fl cult) 27.ix.2002, *Brolio sn* (SP); Itatinga, v.1938, *Lanstyak* sn (HB); Jacupiranga, ii.1962 (ESA); Picinguaba, Castelo Branco road, (fl cult) 27.vi.2003 (SP); São Bernardo do Campo, Alto da Serra, x.1984, (fl cult) 11.xi.2002, *Gil et al. sn* (SP); São José do Barreiro, Núcleo Colonial Senador Vergueiro, Serra da Bocaina (ESA); São Miguel Arcanjo, Carlos Botelho, 31.i.1978, *Prance et al. 6888* (UEC); idem, Parque Estadual de Intervales, 6.xii.2004, *Koehler sn* (UEC); Ubatuba, Picinguaba (SP)

6. *Maxillaria pacholskii* Christenson, Orchid Review 111: 288 (2003) [11]

Type: Ecuador, Oasis, 600 m, *Christenson 2067*, flowered by Ecuagenera (in cult.) in Dec 2002 (holotype, QCNE, n.v.)

Figs. 6A-C and 11

Additional illustrations: Dodson, Native Ecuadorian Orchids, 3: 430, 573, 647 (2002)

[11] Descrição complementada com base em Christenson (2003).

Description: Epiphytes, densely caespitose, up to 5 cm tall. Pseudobulbs cylindrical, ridged, green, 10 x 3 mm, unifoliate. Leaves linear, acute, 32 – 38 x 3 mm. Flowers dark purple, not fragrant. Dorsal sepal linear-elliptic, apiculate, concave, 7 x 3 mm; lateral sepals linear-elliptic, apiculate, 8 x 4 mm. Petals linear-elliptic, abruptly acute, 7 X 2 mm. Lip entire, shallowly arching, with minutely irregular margins, 9 x 5 mm when expanded, the callus oblong. Column arching, abruptly dilated-clavate, unadorned, 7 mm long; foot 3 mm long. Pollinarium with a long stipe, 2-3 mm long.

Distribution and ecology: Ecuador and Peru. Humid forest, 600 m.

Diagnostic characters: Pseudobulbs unifoliate, leaves linear, flower dark red to purple, pollinarium with long stipe, 2-3 mm long.

Comments: Christenson (2003) suggested this species to be closely related to *M. pumila* probably based on general plant architecture. The leaves, however, are more linear and the flowers darker, varying from red to purple, as mentioned by Christenson (2003) himself. Although the author did not describe the pollinarium of this species in the protologue, the analysis of a specimen from cultivation in Ecuador (Ecuagenera) indicated this species has a very similar pollinarium to *M. uncata*, with a very long stipe. DNA sequence data (Koehler et al., unpubl. data; Cap. 1) also support these two species as closely related and suggest the similarity of plant architecture between *M. pacholskii* and *M. pumila* to be convergent.

Specimens examined: In cultivation at Ecuagenera (Gualaceo, Ecuador), *Whitten 2393, s.d.* (FLAS, ESA). **PERU. Pasco.** Villa Rica – Puerto Bermudez, 10°30′ S 75°5′ W, 4.iii.1982, *Gentry & Smith 36076* (MO)

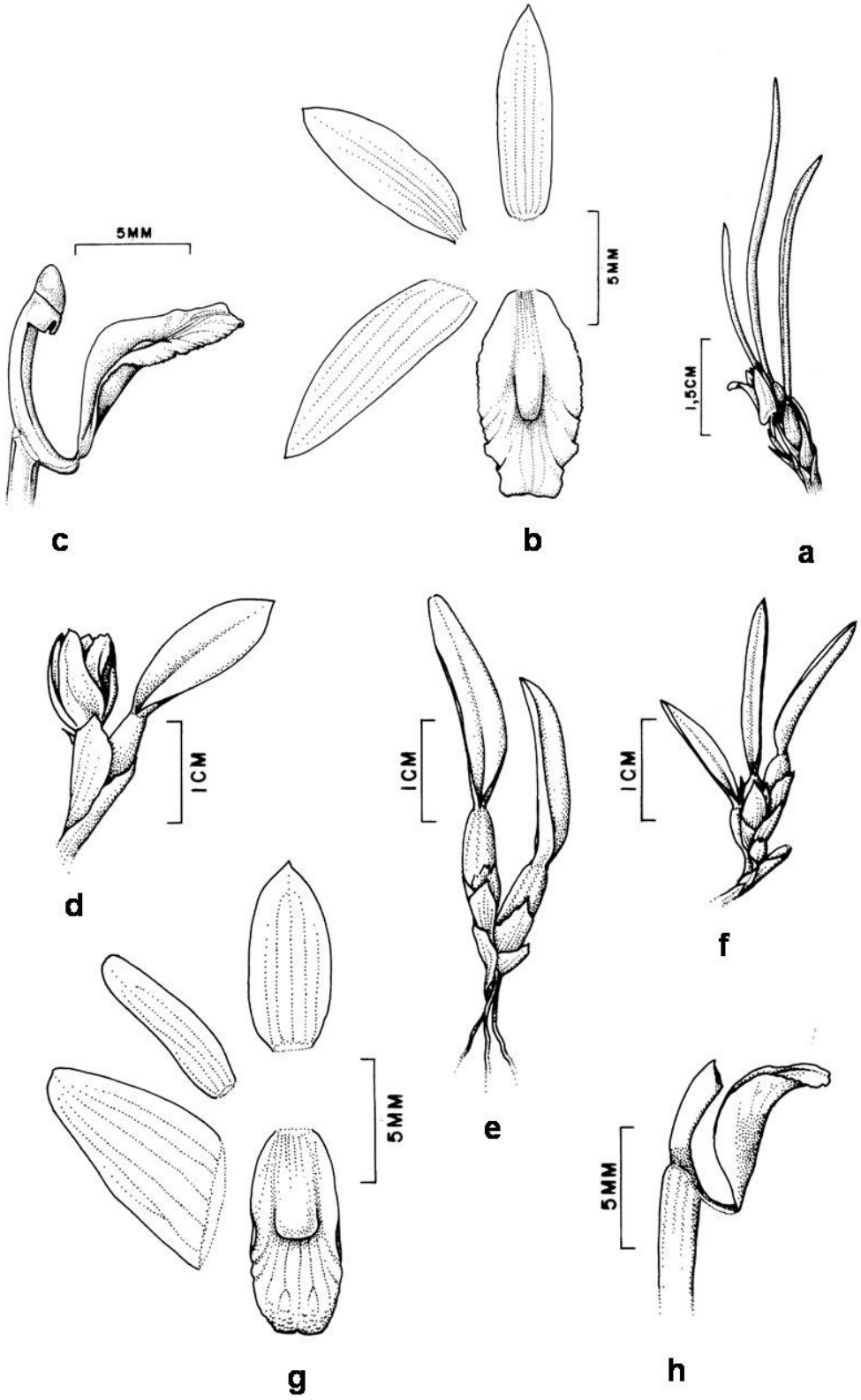


Fig. 6. *Maxillaria pacholskii*. (a) Habit. (b) Flower segments. (c) Column, lateral view with lip attached. *Maxillaria pumila.* (d-f) Habit, showing variation in leaf morphology. (g) Flower segments (h) Column, lateral view with lip attached.

7. *Maxillaria pumila* Hook., Bot. Mag. 64: t. 3613 (1837)

Type: Guyana, Demerara (holotype, missing). Lectotype, here designated: Hooker, *loc. cit.*, t. 3613 (1837).

= *Maxillaria funerea* Lindl., London J. Bot. 1: 188. 1842 **syn. nov.**

Type: Brazil Rio de Janeiro, Praya Vermelha, on dry rocks, *Gardner 123,* (fl) July (holotype, K)

= *Maxillaria plebeja* Rchb. f., Hamburger Garten- Blumenzeitung 15: 57 (1859) **syn. nov.**

= *Maxillaria parva* Rolfe, Bull. Misc. Inform. Kew 193 (1895) **syn. nov.**

= *Maxillaria minuta* Cogn., Fl. bras. (Mart.) 3(6): 68 (1904) **syn. nov.**

= *Maxillaria parahybunensis* Cogn., Fl. bras. (Mart.) 3(6): 64 (1904) **syn. nov.**

= *Maxillaria pusilla* Rolfe, Bull. Misc. Inform. Kew 415 (1908) **syn. nov.**

= *Maxillaria spannagelii* Hoehne, Archiv. Inst. Biol., S. Paulo, 3: 310 (1930) **syn. nov.**

= *Maxillaria minuta* var. *minor* Hoehne, Arq. Bot. Estado São Paulo 2: 136 (1952) **syn. nov.**

Figs. 6D-H and 10A.

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 15(2); Fl. bras. (Hoehne) 10 (12, 7), tab. 156, 157(1), 160 (1953) , Die Orchideen, fig. 1674 (1996)

Description: Epiphytes, 2.5 – 5.5 cm tall, caespitose. Pseudobulbs conical, commonly ridged when mature, dark green, 8 – 15 x 2 – 4 mm, bearing one leaf. Leaves oblong to oblanceolate, fleshy, flat to slightly conduplicate, 18 – 39 x 2 – 7 mm, 1 – 2.5 mm thick. Flowers light red to brownish with segments sometimes yellow at the base, usually without dots, not fragrant, 8 – 10 mm long. Perianth segments never revolute. Dorsal sepal oblong, 6.5 – 9.5 x 2.5 – 4.5 mm; lateral sepals triangular, apiculate, 6.5 – 10.5 x 3 – 5.5 mm. Petals oblong, 6 – 8.5 x 2 - 3 mm, apex rounded. Lip oblong to oblanceolate, 6.5 – 9.5 x 3 – 5 mm when expanded; midlobe apex rounded; callus 2.5 – 5 mm long, clavate. Column 4 – 6 mm; foot 1.5 – 6 mm long. Anther 1 – 2.5 x 1.5 – 2 mm; pollinia 1 x 0.5 – 1 mm; viscidium arched, 0.5 – 1.5 mm long; stipe 0.5 – 1.5 mm. Ovary and pedicel 5.5 – 13 mm long.

Distribution and ecology: Mostly in southeastern Brazil. This species has also been collected in northern Brazil, in the state of Amazonas and Rondônia, although it seems extremely rare in this location. Seasonally dry to wet forest, 0 – 800 m.

Diagnostic characters: M. pumila can be recognised as a small plant with pseudobulbs bearing one coriaceous to fleshy leaf and small dark red to brownish flowers.

Comments: Like for *M. neowiedii,* we propose a more conservative delimitation for the *M. pumila* complex - which traditionally included at least seven species - reducing all morphological variants, described as different species and varieties to synonymy under *M. pumila.* We base our decision on the fact there are no clear morphological characters that allow the identification of the different species traditionally proposed since they vary continuously among taxa. This decision is supported by a phylogenetic study based on DNA sequence (Koehler et al., unpubl. res.; Cap. 1) that support a broad *M. pumila* as a monophyletic group. Also, there are good morphological characters (cited above) that allow the distinction of *M. pumila* from other closely related species.

Specimens examined: **BRAZIL. Amazonas:** rio Japurá, *da Silva sn* (K). **Espírito Santo:** Domingos Martins, 24.viii.1970, *Kautsky 280* (HB); Linhares, Reserva Florestal CVRD, 16.ii.1993, *Folii 1812* (K); Santa Teresa, 10.x.1967, *Emmerich sn* (HB). **Minas Gerais:** Benjamin Constant, 1936, *Horta sn* (RB); Juiz de Fora, Fazenda São Mateus, mata do rio do Peixe, 20.ix.1968, *Vasco Gomes 17* (HB). **Paraná:** Cerro Azul, Morro Grande, 21.xi.1952, *Hatschbach sn* (HB); Guaratuba, Piçarras, xi.1960, *Leinig 217* (HB); Ventania, 4.x.1969, *Seidel 988* (HB). **Rondônia:** rodovia Rondônia, (fl cult) 8.ix.2003 (SP). **Rio de Janeiro:** Carmo, Paquequer river, *Amond 38* (R); Nova Friburgo, Macaé de Cima, xii., *Koehler sn* (UEC); Petrópolis, Araras, caminho do capoeirão, 2.ix.1977, *Martinelli 3094* (RB); Rio de Janeiro, estrada Guanabara, mata do Sumaré, 25.x.1967, *Sucre 1747* (HB); Rio de Janeiro, Guanabara, Dois Irmãos, 4.ix.1952, *Brade 21230* (HB); Teresópolis, cascata Guarani, 2.viii.1958, *Abendroth p-81* (HB); Teresópolis, loteamento Pinheiro, 7.ix.1958, *Abendroth p-87* (HB). **São Paulo:** Bananal, Serra da Bocaina, Morro das Antenas, ix.1994, (fl cult) 6.xii.2002, *Catharino & Ninomya sn* (SP); Barra do Turvo, road BR-116, 8.ii.1995, *Leitão Filho et al. 32874* (UEC); Bertioga, Estação Ecológica da Boracéia, (fl cult) 10.vi.2002, *Neto sn* (SP); Campos do Jordão, 11.xi.2002, *Vinhos sn* (UEC); Guaratuba, Bertioga - São Sebastião road, (fl cult) 16.ix.2003 (ESA); Caraguatatuba, prainha, em pedras, 16.xi.1968, *Pabst 9220* (K); Peruíbe, matas do Clube de Caça e Pesca Garaú, (fl cult) 23.xi.2001, 27.ix.2002 (SP).

8. *Maxillaria subulata* Lindl., Gen. Sp. Orchid. p. 147 (1832)

Type: Brazil, Hort. Loddiges, *s. col.,* Nov. 1818 (holotype, K)

Figs. 7 and 10B

Description: Epiphytes or rupicolous plants, caespitose, 5 – 30 cm tall. Pseudobulbs conical, commonly ridged when mature, dark green, 13 x 48 – 4.4 x 23.5 mm, commonly bearing two, more rarely one, leaves. Leaves very variably, subulate to lanceolate, membranaceous to coriaceous, flat and commonly twisted or aciculate to cylindric, strongly conduplicate and usually straight, but also recurved, (36-) 50 (-235) x 1 - 14 mm, 0.5 – 2 mm thick. Flowers reddish, sometimes with yellow regions at the base of perianth segments, or tan-colored to brownish, rarely pale yellow, usually with dark red dots and a strongly fruity, watermelon like fragrance, 11 – 23 mm long. Perianth segments commonly revolute. Dorsal sepal oblong, 12 – 23 x 3 – 8.5 mm; lateral sepals ovate to triangular, apiculate, 10 – 22 x 2.5 – 10 mm. Petals oblong to oblanceolate, 10 – 20 x 2.5 – 9 mm, apex acute or rounded. Lip obovate to oblanceolate, 10 x 19 mm when expanded; midlobe orbicular, apex rounded, sometimes retuse; callus 4.5 – 9.7 mm long, oblong. Column 6 – 13.5 mm, foot 2.5 – 7 mm long. Anther 2 – 4.5 x 1.5 – 4 mm; pollinia 1 – 1.5 –x 1 – 2.5 mm; viscidium arched, 1.5 – 4 mm long; stipe 1 – 5 mm long. Ovary and pedicel 12 – 28.5 mm long, always shorter than adjacent pseudobulb.

Distribution and ecology: This is one of the most widespread and polymorphic species of this group, occurring from Santa Catarina to southern Bahia in seasonally dry to wet Atlantic forest and *campos rupestres* formations, 0 – 2200 m.

Maxillaria acicularis, M. madida, M. mosenii and *M. subulata* were traditionally recognized as distinct species based on vegetative and floral characters. However, like *M. neowiedii* and *M. pumila,* species limits within this complex were not clear due to the continuous variation of the morphological diagnostic characters. Detailed studies using AFLP data (S. Koehler et al., unpubl. res., (Cap. 3) indicated that a single species and four varieties should be recognized for this complex. This broadly defined species could be recognized by the following characters: pseudobulbs conical, commonly ridged when mature, bearing two, rarely one, acicular, cylindrical or lanceolate leaves, commonly longer than 50 mm; ovary and pedicel always shorter than adjacent pseudobulb; flowers usually with strong fruity fragrance, dark red to brownish, usually spotted; petals oblanceolate with an acute apex; lip with an oblong callus.

8a. *Maxillaria subulata* Lindl. var. *subulata*

= *Maxillaria echinochila* Kraenzl., Ark. Bot. 16(8): 22 (1921). ≡ *Maxillaria mosenii* var. *echinochila* (Kraenzl.) Hoehne, Arq. Bot. Estado São Paulo 2: 73 (1947) **syn. nov.**

Type: Brazil, Paraná, Cadeado, *Dusén 7012*, 2 Nov 1908 (holotype, S, n.v.)

Additional illustrations: Fl. bras. (Hoehne) 10 (12, 7), tab. 153 (misidentifed as *M. madida*), 154 (misidentified as *M. madida* Lindl. var. *pallida* Cogn.) (1953)

Description: Rupicolous plants. Pseudobulbs bifoliate. Leaves cylindrical and fleshy. Flowers with strongly fruity, watermelon like fragrance

Distribution and ecology: This variety is mainly restricted to the *campos rupestres* formations in the state of Minas Gerais, but can be found ocasionally as epiphytes in humid and seasonally dried forests in the state of Espírito Santo.

Specimens examined: **BRAZIL. Espírito Santo:** Itaguassú, Alto Limoeiro, 17.v.1946, *Brade et al. 18546* (RB). **Minas Gerais:** Bom Jardim de Minas, *Saleh 79* (HB); Caeté, Serra da Piedade, 18.i.2004, *Koehler sn* (UEC); Datas – Diamantina, *Windisch 2609* (HB); Mogol, (fl) 12.viii.2003 (ESA); Nova Lima, Serra do Curral, 30.iii.1945, *Williams & Assis 6494* (R, RB); Ouro Preto, Pico do Itacolomi, (fl cult) 23.ix. 2003, *Eunice sn* (ESA); Presidente Jucelino, 1978, (fl cult) 5.viii.2003, *Bicalho sn* (ESA); Rio de Janeiro-Belo Horizonte road, mineração Harriaca, (fl cult) 10.vii.2004 (ESA); Santa Bárbara, Serra do Caraça, 26-30.v.2004, *Custodio et al.* (UEC); Santana do Riacho, Serra do Cipó, 18.vi.2002, *Koehler & Aranha sn* (UEC)

8b. *Maxillaria subulata* Lindl. var. *angustipetala* (Hoehne) S. Koehler, ≡ *Maxillaria madida* var. *angustipetala* Hoehne. Arq. Bot. Estado São Paulo n.s. 2: 136 (1952) **comb. nov.**

Type: Brazil, Rio de Janeiro, Petrópolis, *C. Spannagel 188, s.d.* (holotype, SP)

= *Maxillaria acicularis* Herb. ex Lindl., Edwards's Bot. Reg. 23, sub tab. 1986 (1837)

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 16(2); Fl. bras. (Hoehne) 10 (12, 7), tab. 150(3), 158(1) (1953)

Description: Epiphytes generally found in the canopy, very rarely rupicolous plants. Pseudobulbs bifoliate. Leaves aciculate. Flowers commonly not fragrant

Distribution and ecology: Found in wet Atlantic forest formations, usually in the tree canopy, but also in riparian forests.

Comments: It is extremely difficult to distinguish between young plants of *M. subulata* var. *angustipetala* and young plants of *M. subulata* var. *subulata.* The former usually comprises more delicate plants with smaller, non-fragrant flowers. Also plants of *M. subulata* var. *angustipetala* generally grows as epiphytes and are rarely found as a rupiculous plant while plants of *M. subulata* var. *subulata* grow mainly on rocks.

Specimens examined: **BRAZIL. Espírito Santo:** Castelo, 27.xi.1972, *Seidel 1055* (RB); Domingos Martins, 13.iii.1964, *Kautsky 13* (HB); Linhares, Reserva Florestal Vale do Rio Doce, 3.vi.2003, *Koehler & Singer sn* (UEC); Santa Teresa, 23.viii.1941, *A. Ruschi* 29 (R); Vargem Alta, 1949, vii.1951, *Brade 272* (RB). **Minas Gerais:** Benjamin Constant, 1935, vii.1936, *Horta sn* (RB); Bom Jardim de Minas, 1.V.1962, *Saleh 93* (HB); Descoberto, Serra do Descoberto, *Schwacke 11125* (RB); Tiradentes, Serra de São José, 15.v.1998, *Becker & Alves 314* (RB). **Rio de Janeiro:** Mocotó-Poço Parado, 6.vii.1962, (fl cult) vii.2003, *Oliveira sn* (ESA); Nova Friburgo, Macaé de Cima, 20.vii.2003, *Koehler & Pinheiro sn* (UEC); Poço Parado – São Fidelis, 6.vii.1962, (fl cult) vii.2003, *Oliveira sn* (ESA); Rio de Janeiro, Serra da Carioca, Sumaré road, 22.iv.1951, *Pabst 1041* (HB); Rio de Janeiro, Tijuca, Pedra do Conde, x.1949, 4.iv.1950, *Pabst 395* (RB); Santa Maria Madalena, vii.1969, *Ventura sn* (HB); Teresópolis, cascata Guarani, 9.xi.1958, *Abendroth p-112(222)* (HB)

8c. *Maxillaria subulata* var. *madida* (Lindl.) S. Koehler. ≡ *Maxillaria madida* Lindl., Bot. Reg. 74, misc. 44 (1838) **comb. nov.**

Type: Brazil, *s. loc., s.d.* (holotype, K)

= *Maxillaria mosenii* Kraenzl., Kongl. Svenska Vetenskapsakad. Handl. 46(10): 73 (1911). **syn. nov.**

= *Maxillaria hatschbachii* Schltr., Repert. Spec. Nov. Regni Veg. 23: 56 (1926). ≡ *Maxillaria mosenii* var. *hatschbachii* (Schltr.) Hoehne, Arq. Bot. Estado São Paulo 2: 73 (1947) **syn. nov.**

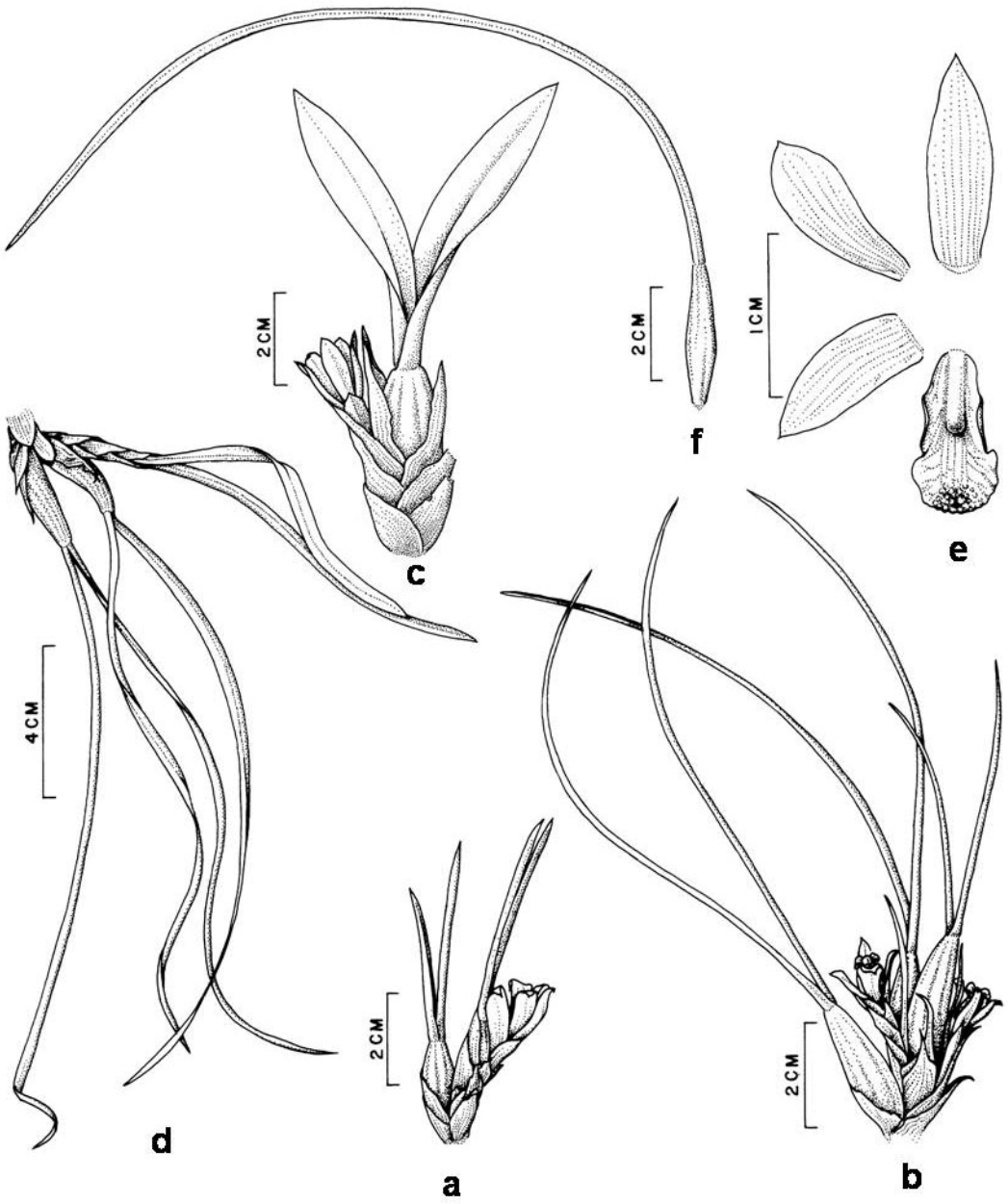


Fig. 7. *Maxillaria subulata* var. *subulata.* (a) Habit. *M. subulata* var. *acicularis.* (b) Habit. *M. subulata* var. *madida.* (c-d) Habit, showing variation in leaf morphology. (e) Flower segments. *M. subulata* var. *monophylla.* (f) Habit.

= *Maxillaria paulistana* Hoehne, Arq. Bot. Estado São Paulo 2: 136 (1952) **syn. nov.**

[*Maxillaria mosenii* var. *typica* (Hoehne), Fl. Bras. 12 (7): 310 (1953) **nom. inval.**]

Additional illustrations: Fl. bras. (Hoehne) 10 (12, 7), tab. 144, 145, 146 (1953)

Description: Epiphytes, rarely rupicolous plants. Pseudobulbs bifoliate, rarely unifoliate. Leaves subulate to lanceolate, membranaceous to coriaceous, flat and commonly twisted, and fleshy. Flowers with strongly fruity, watermelon like fragrance.

Distribution and ecology: By far the most common and widespread variety of *M. subulata*. It is found in wet and seasonally dry Atlantic forest formations from Bahia to Santa Catarina, usually in the tree canopy.

Comments: The analysis of herbarium specimens as well as cultivated plants from many different localities showed that the different species placed here under synonymy of *M. subulata* var. *madida* (see review in Hoehne 1953) belong to a continuous range of morphological variation. *Maxillaria paulistana,* described as having unifoliate pseudobulbs and an intermediate morphology between *M. heterophylla* var. *heterophylla* and *M. pachyphylla* (Hohne 1952) fits the circunscription of *M. subulata* var. *madida*, with flat, lanceolate leaves and flowers with pedicel and ovary length smaller than the adjacent pseudobulb. Unifoliate specimens of *M. subulata* var. *madida* are rare, but have been observed in cultivation. There is not enough data available to access if this character is restricted to individuals or to whole populations.

Specimens examined: **BRAZIL. Bahia:** Ilha de Itaparica, (fl cult) 1.viii.2003 (ESA. **Espírito Santo:** Muqui, Fazenda Santa Maria, 27.vi.1958 (fl cult) 1.v.2003, *Oliveira sn* (ESA). **Paraná:** Antonina, Cotia river, 29.xi.1955, *Hatschbach* 13181 (HB); Guanabara, road to Joinville, 2.xi.1952, *Hatschbach sn* (HB); Curitiba, Serra Negra, 1961, (fl cult) 2.xii.2003 (ESA); Marumbi, 15.i.1959, *Leinig 82* (HB); Toledo, Bom Princípio, ix.1959, *Leign 147* (HB); Vassoroca, 25.i.1955, *Nestor sn* (HB). **Rio de Janeiro:** Angra do Reis, Fazenda Pedra Branca, 27.i. 1962, (fl cult) v.2003, *s. col.* (ESA); Cunha-Parati, Serra do Mar, vertente do Parati, xi.1966, *Hoehne sn* (SP); Itatiaia, 25.xi.1941, *Baumgart sn* (RB); Mauá-Itatiaia, morro do mata-cavalo, iii.1965, (fl cult) 1.viii.2003, *Oliveira sn* (ESA); Rio Bonito, Itatiaia, (fl cult) 2.vii.2003 (ESA). **Santa Catarina:** Florianópolis, iii.1950, *Rohr 2103* (HB); Orleans, propriedade Rio Novo, vii.1962, (fl cult) 8.ix.2003, *Bicalho & Targa sn* (SP); Palhoça, morro do Cambirela, 20.x.1971,

Klein & Bresolin 9843 (HB); Pântano do Sul, 11.iii.1950, *Rober 2103* (HB). **São Paulo:** Apiaí-Iporanga, 24.ix.1960, (fl cult) 2.xii.2003, *Oliveira sn* (ESA); Atibaia, Parque Municipal da Grota Funda, *Bernacci et alli 28433* (UEC); Bananal, Serra da Bocaína, sertão do rio Vermelho, iv.1936, vi.1936, *Brade sn* (HB); Boracéia, (fl cult) 8.ix.2003 (SP); Cananéia, Ilha do Cardoso, 24.i.2003, *Breier sn* (UEC); Capão Bonito, Fazenda Boa Esperança, 28.viii.1961, (fl cult) 1.viii.2003, *Oliveira sn* (ESA); Guapiara, Fazenda Brasil, ii.1961, (fl cult) 1.viii.2003, *Oliveira sn* (ESA); Guaratuba, Bertioga-São Sebastião road, (fl cult) 1.viii.2003, *Feldmann sn* (ESA); Jacareí-Ribeirão Claro, Reserva Florestal Sra Perches, x. 1961, (fl cult) ix.2003 (ESA); Jacupiranga, xi.1964, (fl cult) 8.ix.2003, *Bicalho sn* (ESA); Maresia, Bertioga-São Sebastião road, (fl cult) v.2003 (ESA); Mogi das Cruzes, Boracéia, Reserva DAE, 22.xii.1966, (fl cult) 1.viii.2003, *Martins sn* (ESA); Moji-Bertioga road, 21.vii.1982, *Tinoco & Tinoco sn* (SP); Paranapiacaba, 23.vi.1981, (fl cult) 24.iii.2003, *Gil et al. sn* (SP); Paranapiacaba, Parque Estadual Carlos Botelho, 17-20.vii.1967, (fl cult) 16.i.2003, *Hoehne sn* (SP); Pilar do Sul, Fazenda Brasil, 25.viii.1960, (fl cult) 1.viii.2003, *Oliveira sn* (ESA); Salesópolis-Caraguatatuba, Serra do Juquesi, x. 1988, *Catharino sn* (SP); São José do Barreiro, Serra da Bocaina, 21.vii.1994, (fl cult) 1.xi.2001, *Catharino sn* (SP); São Paulo, Engenheiro Marsilac Parelheiros, 18.i.1996, (fl cult) 10.vi.2002, *Garcia 753* (SP); São Paulo, Instituto de Botânica de São Paulo, 30.iii.1965, (fl cult) 8.ix.2003, *Texeira sn* (SP); São Paulo, interligação Imigrantes-Anchieta, Serra do Mar, 6.vi.1988, (fl cult) 10.vi.2002, *Brolio et al sn* (SP); Serra da Bocaina, (fl cult) 2.vii.2003 (ESA)

8d. *Maxillaria subulata* var. *monophylla* (Cogn.) S. Koehler. ≡ *Maxillaria madida* Lindl. var. *monophylla* Cogn. Bull. Soc. Roy. Bot. Belgique 43: 330 (1906) **comb. nov.**

Type: Brazil, Rio de Janeiro, *Glaziou 20502, s.d.* (holotype, not found).

Additional illustration: Butzin & Senghas, Schltr. Die Orchideen, fig. 1671 (1996)

Description: Rupicolous, rarely epiphitic plants. Pseudobulbs unifoliate. Leaves cylindrical, commonly recurved. Flowers fragrant.

Distribution and ecology: This variety is mainly restricted to the states of Minas Gerais and Bahia, with the exception of the holotype, which was collected in the state of Rio de Janeiro.

Specimens examined: **BRAZIL. Bahia:** Floresta Azul, Fazenda Indaiá, (fl cult) 16.ix.2003, *Oliveira sn* (ESA); Pedras, em mangue, 25.x.1965, (fl cult) 1.viii.2003, *Oliveira sn* (ESA); Porto Seguro, Fazenda

São José, (fl cult) 10.xii.2004 (ESA); Santa Luzia, rio Salgado, (fl cult) 10.xii.2004 (ESA); Una, Fazenda dos Belgas, 25.x.1963, (fl cult) 2.xii.2003, *Oliveira sn* (ESA). **Minas Gerais:** Coronel Pacheco, Estação Experimental, *Vasco Gomes sn* (HB)

9. *Maxillaria uncata* Lindl., Edwards's Bot. Reg. 23: sub tab. 1986 (1837). ≡ *Camaridium uncatum* (Lindl.) Hoehne, Arq. Bot. Estado São Paulo 2: 127 (1952)

Type: Guiana, Demerara, *Loddiges s.n., s.d.* (holotype, K)

= *Maxillaria nana* Hook., Icon. Pl. 4: tab. 315 (1841)

= *Maxillaria stenostele* Schltr., Beih. Bot. Centralbl. 36(2): 414 (1918)

= *Maxillaria subulifolia* Schltr., Repert. Spec. Nov. Regni Veg. Beih. 7: 172-173 (1920) **syn. nov.**

= *Maxillaria squamata* Barb. Rodr., Gen. Sp. Orchid. 1: 118 (1877). ≡ *Ornithidium squamatum* (Barb. Rodr.) Barb. Rodr. in Gen. Sp. Orchid. 2: 209 (1882). ≡ *Camaridium squamatum* (Barb. Rodr.) Hoehne in Arq. Bot. Estado São Paulo 2: 72. 1947.

= *Maxillaria striatella* Kraenzl., Repert. Spec. Nov. Regni Veg. 24: 359 (1928).

Figs. 8A-D and 11

Additional illustrations: Mori et al., Mem. New York Bot. Gard., p. 315 (1997); Bennett & Christenson, Ic. Orch. Peruv., pl. 117 (1993); Butzin & Senghas, Schltr. Die Orchideen, fig. 1672 (1996); Atwood & Mora de Retana, Fieldiana Bot. n.s. 40: fig.13F.; Bennett & Christenson, Ic. Orch. Peruv., pl. 710 (2001); Dodson, Native Ecuadorian Orchids, 3: 430, 573, 647 (2002)

Description: Epiphytes, repent with shoots up to 2.5 cm apart or caespitose. Pseudobulbs cylindrical, smooth, glossy green, 5 – 30 mm long, unifoliate. Leaves elliptic to oblong, sometimes linear, fleshy, flat to terete, 15 – 100 x 1 – 20 mm, apex acute. Flowers waxy white to light purple, commonly with pinkish stripes, rarely light reddish, not fragrant, 10 - 15 mm long. Dorsal sepal elliptic-lanceolate, elongate, 7 – 14 x 2 - 5 mm; lateral sepals ovate, triangular, apiculate, 9 – 15 x 2 – 5 mm. Petals lanceolate, elongate, 6 – 15 x 1 – 5 mm, apex acute. Lip oblong to elliptic-ligulate, 9 – 19 x 2 – 4 mm when expanded, slightly denticulate; midlobe obtuse; callus 4 – 5 mm long, oblong to clavate. Column 5 - 14 mm; foot usually almost as long as column, 4 – 12 mm long. Anther 1.5 x 1 – 1.2 mm; pollinia

0.5 – 0.7 x 0.3 – 0.5 mm; viscidium arched, 1 mm long; stipe 2 – 2.5 x 0.5 – 0.7 mm. Ovary and pedicel 7 – 20 mm long, longer than adjacent pseudobulb

Distribution and ecology: From Chiapas (Mexico) and the West Indies to the Amazon region in Northern and Northwestern South America down to Bolivia. Widespread. Seasonally dry and wet forest formations, 50 – 1700 m.

Diagnostic characters: M. uncata can be distinguished from other species from this group by its unifoliate pseudobulbs with fleshy leaves; by the waxy white to light purple flowers with an elliptic-lanceolate, elongate perianth and by the pollinarium with a long stipe (2-3 mm).

Comments: M. uncata comprises a widespread and highly polymorphic species and previous authors (Atwood & Mora de Retana, 1999; Bennett & Christenson, 2001) have suggested there may be more than one species. However, further morphological and molecular studies are necessary in order to understand patterns of diversity within this group. For this treatment we accepted the current taxonomic delimitation adopted by Atwood & Mora de Retana (1999) and Bennett & Christenson (1993, 2001) and reduced *M. subulifolia* under synonymy of *M. uncata* based on the drawing of the type deposited at AMES, which agrees with the current broad circunscription of *M. uncata*.

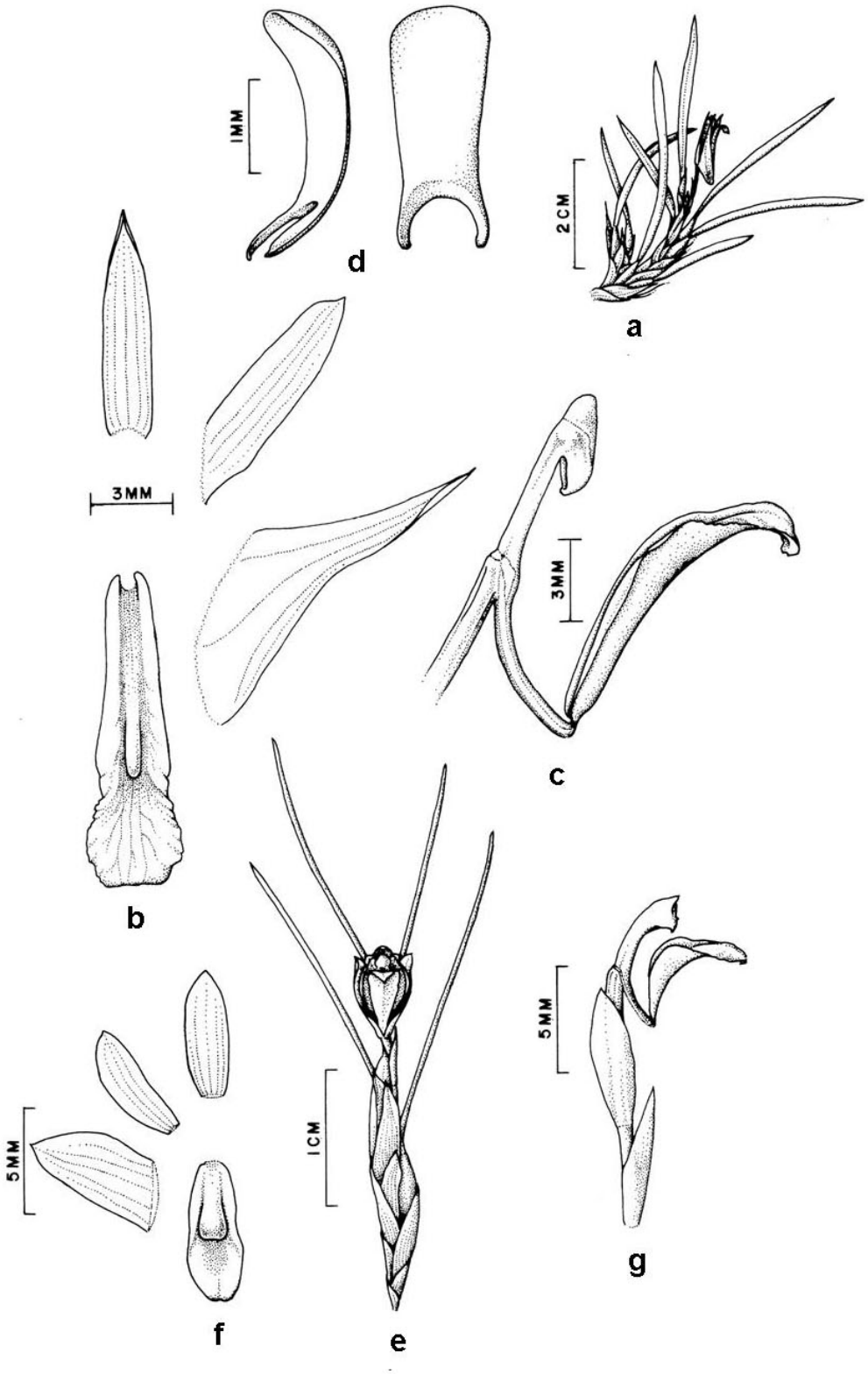


Fig. 8. *Maxillaria uncata*. (a) Habit. (b) Flower segments. (c) Column, lateral view with lip attached. (d) Pollinarium without pollinia. *Maxillaria vernicosa*. (e) Habit. (f) Flower segments. (g) Column, lateral view with lip attached.

Specimens examined: **BELIZE. New Town:** 11.viii.1932, *Schipp 916* (K). **BOLIVIA. Pando:** west bank of Madera river, 23.vii.1968, *Prance et al. 6267* (K, NY). **BRAZIL. Amapá:** Rio Oiapoque, near Cachoeira três Saltos, 2º12′ N 52º53′ W, 10.vii.1960, *Irwin et al. 48139* (NY). **Amazonas:** Ilha Aramaçá, almost opposite Tabatinga, 24.vii.1973, *Prance et al. 16810* (NY). Itapiranga, rio Uatama, 26.viii.1979, *Cid et al. 828* (K); Manaus-Itacoatiara road Km 26, Reserva Ducke, 2º53′S 59º58′W, 13.ii.1996, *Ribeiro et al. 3/96* (K). **Maranhão:** Zé Doca, km 180 da BR 316, km 13 de acesso, 12.xii.1978, *Rosa & Vilar 2935* (NY). **Roraima:** Ilha de Maracá, SEMA Ecological Reserve, 3º43′ N 62º41′W, 22.v.1987, *Milliken & Bowles sn* (K). Serra da Lua, 2º25′-29′ N 60º11-14′ W, 17.i.1969, *Prance et al. 9331* (NY). **Pará:** Cuanta do Anajás and Vista Alegre, 0º57′ S 49º48′ W, 4.xi.1987, *Prance et al. 30287* (NY). Basin of Rio Trombetas, Km 109, access road from Cachoeira Porteira to Perimetral de Norte, 26.vi.1974, *Campbell et al. P22278* (NY). Rio Jarí, Planalto Monte Dourado, 26.i.1968, *E. de Oliveira 3997* (NY). Sete Varas airstrip on rio Curua, 0º95S 54º92W,10.viii.1981, *Strudwick & Sobel 4440* (K). **COLOMBIA. Antioquia**: 1884 (K); Anori, Buenes Aires, ca 4 Km from Providencia, 12.xii.1972, *Soejarto & Rentería 3632* (MO); Turbo, Carretera Tapón del Darién, sector Río León-Lomas Aisladas, Km 38, 29.ii.1984, *Brand & González 982*. **Chocó:** Riosucio, Parque Natural Nal, Los Katyos, Salto de Tilupo, 19.vi.1976**,** *Léon 108* (MO). **Cali**: west Andes, 1906, *Lehmanniane 8135* (K). **Santander:** between El Roble and Tona, 17.ii.1927, *Killip & Smith 19432* (NY). **Valle:** Bajo Calima, 3º55′ N 77º2′W, 10.7.1984 (MO). **COSTA RICA. Alajuela**: San Carlos, La Fortuna, Finca El Jilguero, 10º26′10′′N 84º42′20′′W, 21.xi.1992, *Herrera 5556* (K, US). **Cartago:** Pejivalle, i.1940, *Skutch 4624* (US). **Guanacaste:** Parque Nacional Guanacaste, La Cruz, 10º59′ 26′′N 85º25′40′′W, 24.x.1990, *Chavez 222* (K). **Limón:** La Colombiana Farm of the United Fruit Co, 6-7.iii.1924, *Standley 36910* (US). **Puntarenas:** 37 km W of San Vito de Java on the main roas towards Carretera Interamericana, 10.i.1992, *Grant 92-01831 & Rundell* (US). **ECUADOR**. *HK 980* (K). **Napo:** Yasuni National Park, 21.i.1988, *Dodson & Niell 17363* (K). **Pastaza:** Pastaza Cantón. 4-21.x.1990, *Gudiño et al. 1054* (MO). **FRENCH GUYANA.** Karouany, 1856, *Sagot 869* (K); Savane Roche du Quatorze Juillet – Bassin du Bas-Oyapock, 3º58′ N 51º52′ W, 16.iv.1991, *Cremers 12171* (MO). **Cayenne:** 50 km of Cayenne on the road to Regina after the turnoff to Cacao, 5.ix.1968, *Kress & Stone 88-2518* (US). Camp Eugène – Bassin du Sinnanary, 4º 51º N 53º 4′ W, 6.ii.1995, *Cremers & Granville 13700* (MO). **Saul:** on route de Belizon north of Eaux Claires, 3º47′ N 53º W, 8.xi.1990, *Mori et al. 21563* (US). **GUATEMALA. Peten:** Dolores, 29.vii.1961, *Contreras 2998* (US). **GUYANA.** Barima river, 1898, *Thurn 89* (K). U. Demerara – Berbice, Mabura Hill, 5º20′ N 58º40′ W, 4.vi.1994, *Christenson 1893 et al.* (US). U. Takutu – U. Essequibo Region, 20 km NE of Surama Village, 4º15′ N 59º56′ W, 1.iii.1990, *McDowell 2095 et al.* (NY). **Bartica**: Esequibo river, 17.viii.1929, *Sandwith sn* (K); Cuyuni-Mazaruni**,** 0.8 S of Eping River camp, 6º05′ N 60º07′ W, 10.xi.1990 (US). **Mabura:** log catchment, Kurupukari main road km 12,

5º20′ N 58º 30′ W, 26.xi.1992, *Ek 589* (MO). **Mazaruni-Potaro:** Kaieteur Falls, 1937, *Sandwith 1281* (K); Kanuku mountains, 4-22.iii.1938, *Smith 3292* (K); Mazuruni, 5º41′30′′N 60º40′30′′W, 6.iv.1979, *Edwards 1179* (K); Mazuruni Station, 27.viii.1937, *Sandwith 1222* (K). **HONDURAS. Comayagua:** Meambar, 31.vii.1933, *Edwards 471* (K). **MEXICO: Chiapas:** near Laguna Ocotal Grande, ca. 25-30 km SE of Monte Líbano, 20.vi – 20.viii.1954, *Dressler 1456* (US). **NICARAGUA. Chontales:** vi.1868, *Tate 220* (K). **PANAMA.** 1907, *Munch sn* (NY). **Cocle:** Rio Cocle del Norte, prepared on 1.xii.1960, *Dunn 16* (NY). **San Juan:** canal zone, viii-ix.1920, *Powell 120* (K). **PERU. Huánuco:** Leoncio Prado, Rupa Rupa, Jacintillo, margen izquierda del Río Monzón, 12.xi.1971, *Schunke 5145* (NY, US). **Loreto:** Punchana, Maynas, 10.i.1954, *McDaniel 32114 & Rimachi* (MO). **PUERTO RICO.** cultivated at USDA Agricultural Station at Mayaquez, 2.xi.1951, (fl) 21.ii.1967, *Hutchinson 6* (K). **SURINAME.** Wihelmina Geberte, 3º36′-3º41′N 56º30′-56º34′W, 25.viii.1963, *Irwin et al. 55063* (K, NY). **Brokopondo:** 21.iii.1966, *van Donselaar 3229* (K). 2 km S of Afobaka, ii.1965, *van Donselaar 2114* (NY). **VENEZUELA. Amazonas:** Atabapo, 4º30′N 65º48′W, x.1989, *Delgado 771* (MO). Atures, 5º41′N 66º28′W, 28.ii.3207, *Holst & Liesner 32907* (MO). **Bolivar:** along trail east of pica 101, 5-7 km E of El Cruzero, ESE of Villa Lola, 16.vii.1960, *Steyermrk 86410* (NY). **Delta Amacuro:** Rio Cayubini, along lower section of the river, upstream from Casa Cuyubini, 12.xi.1960, *Steyermark 87483* (NY). **Rio Negro:** Río Siapa near base of Cerro Aracamuni, 1º39′ N 65º40′ W, 4.xi.1987, *Liesner & Carnevali 22783* (MO)

10. ***Maxillaria vernicosa*** **Barb. Rodr.,** Gen. Sp. Orchid. Nov. 1: 121 (1877)

Type: Brazil, Minas Gerais, Santa Rita do Rio Claro, *J. Barbosa. Rodrigues s.n.*, Mar (holotype, missing). Lectotype, here designated: Barbosa Rodrigues J., Iconographie des Orchidées du Brésil, tab. 291, vol. 6, reproduced by Sprunger et al. (1996).

= *Maxillaria vitelliniflora* Barb. Rodr., Gen. Sp. Orch. Nov. 1: 121 (1877)

Figs. 8E-G and 10C

Additional illustrations: Fl. bras. (Mart.) Vol. 3(6), tab. 7(2,3); Fl. bras. (Hoehne) 10 (12, 7), tab. 159(2) (1953), Butzin & Senghas, Schltr. Die Orchideen, fig. 1670 (1996)

Description: Epiphytes, 2 – 5 cm tall, caespitose. Pseudobulbs conical, commonly ridged when mature, light to dark green, 8.5 – 13 x 2 – 3 mm, bearing two leaves. Leaves aciculate, cylindrical, 12.5 – 30.5 x 0.4 – 0.6 mm. Flowers vivid yellow, sometimes red to purple at the

base of perianth segments, never with dots, not fragrant, 6 – 8.5 mm long. Flower segments never revolute. Dorsal sepal oblong, 5.5 – 8.5 x 2 – 3 mm; lateral sepals ovate to triangular, apiculate, 6 – 8.5 x 2.5 – 6.5 mm. Petals oblanceolate, 5 – 7.5 x 1 – 2.5 mm, apex rounded. Lip oblong, 5.5 – 7.5 x 2 – 4 mm when expanded; midlobe apex rounded and retuse; callus claviform or retuse, 3 – 5 mm long. Column 2.5 – 5.5 mm; foot 1 – 3.5 mm long. Anther 1 – 1.5 x 1 – 1.5 mm; pollinia 1 x 1 mm; viscidium pad-like, 1 mm long; stipe 0.8 – 1 mm. Ovary and pedicel 12 – 19 mm long, always longer than adjacent pseudobulb

Distribution and ecology: South to southeastern Brazil, except in the state of Espírito Santo. Seasonally dry to wet forest formations, 200 – 1700 m.

Diagnostic characters: M. vernicosa differs from other species of this group by its small size, up to 5 cm tall, its pseudobulbs bearing two apiculate leaves, and by its vivid yellow, tiny flowers.

Comments: Barbosa Rodrigues (1877) described two species*; M. vernicosa* and *M. vitelliniflora*, but never published the illustrations, which were reproduced by Sprunger et al. (1996). Although Barbosa Rodrigues (1877) considered these two species very similar, he separated them based only on the colouration of flower segments (perianth and anther). We do not consider this to be a good character to distinguish these two species, since the colouration of flower segments varies slightly among populations, mainly the base of the perianth segments, as observed in cultivated specimens during this study.

Specimens examined: **BRAZIL. Bahia:** Castro Alves, Fazenda Garrão José Rodrigues (SP). **Minas Gerais:** Baependi, 27.ii.1941, *Carvalho sn* (RB); Bom Jardim de Minas, 15.iii.1961, *Saleh 44* (HB); Caldas (SP), (fl cult) 10.vi.2002, 24.iii, 2003 (SP); Camanducaia, Fazenda Levantina e Vila Monte Verde, (fl cult) 16.i.2003 (SP); Caxambú, vii.1950, (fl cult) 16.ii.1951, *Brade & Apparicio 20588* (RB); Varginha, 23.iii.1954, *Welter* 104 (HB). **Paraná:** Arapoti, rio Perdizes, 6.iv.1970, *Hatschbach 24113* (HB); Campinas Grande do Sul, sítio do Belizário, 26.ix.1969, Hatschbach 22226 (HB); Catanduvas, 5.xii.1969, *Hatschbach & Ravenna* 23131 (HB); Curitiba – Rio Negro road km 21, rio Iguaçú, 10.xi.1959, *Leinig 164* (HB); Curitiba, iii.1944, *Guimarães sn* (RB); Foz do Iguaçu [Santa Maria do Salto], rio Iguaçu, 19.xi.1920, *Lange sn* (HB); Francisco Beltrão, Salto Santo Rosa, xi.1971, *Leinig 478* (HB); Itaiacoca, iii.*1904, Dusén 4243* (RB); Laranjeiras do Sul. Salto Osório, rio Iguaçu, 15.xi.1968, *Hatschbach 20345* (HB); Matinhos, 21.xii.1944, *Guimarães sn* (RB); Quatro Barras, Florestal, 2.iii.1969,

Hatschbach 21207 (HB); Ribeirão Grande, Curitiba – São Paulo road, iii.1964, *Leinig 319* (HB); Roca Nova, Serra do Mar, xii.1958, *Leinig 66* (HB); Toledo, Bom Princípio, ix.1959, *Leinig 148* (HB). **Rio Grande do Sul:** Canela, 20.iii.1953, *Pabst 628* (HB); Gramado, iii.1950, *Pabst sn* (HB); São Francisco de Paula, 20.xi.1952, *Welser sn* (HB). **Rio de Janeiro:** Petrópolis, Correas, 24.xi.1974, *Mello* 3a (HB); Rio de Janeiro, matas da Tijuca, ix.1956, *Pabst sn* (HB). **Santa Catarina:** Florianópolis, Naufragados, 17.xi.1970, *Klein & Bresolin* 9209 (HB); Florianópolis, Tapera, Ribeirão, morro da Cotia, 14.x.1969, *Klein & Bresolin 8364* (HB); Florianópolis, viii.1950, *Rohr 793* (HB); Ibirama, Horto Florestal, 11.ii.1953, *Reitz & Klein 1147* (HB); Lauro Müller, Vargem Grande, 24.x.1958, *Reitz & Klein 7469* (HB); Nova Teutonia, 5.xi.1944, *Plaumann 604* (RB); Rio do Sul, Matador, 31.xii.1958, *Reitz 6173* (HB); Tijucas, Pinheiral, 2.xi.1950, *Rohr 2025* (HB). **São Paulo:** Campos do Jordão, *Kuhlmann sn* (HB)

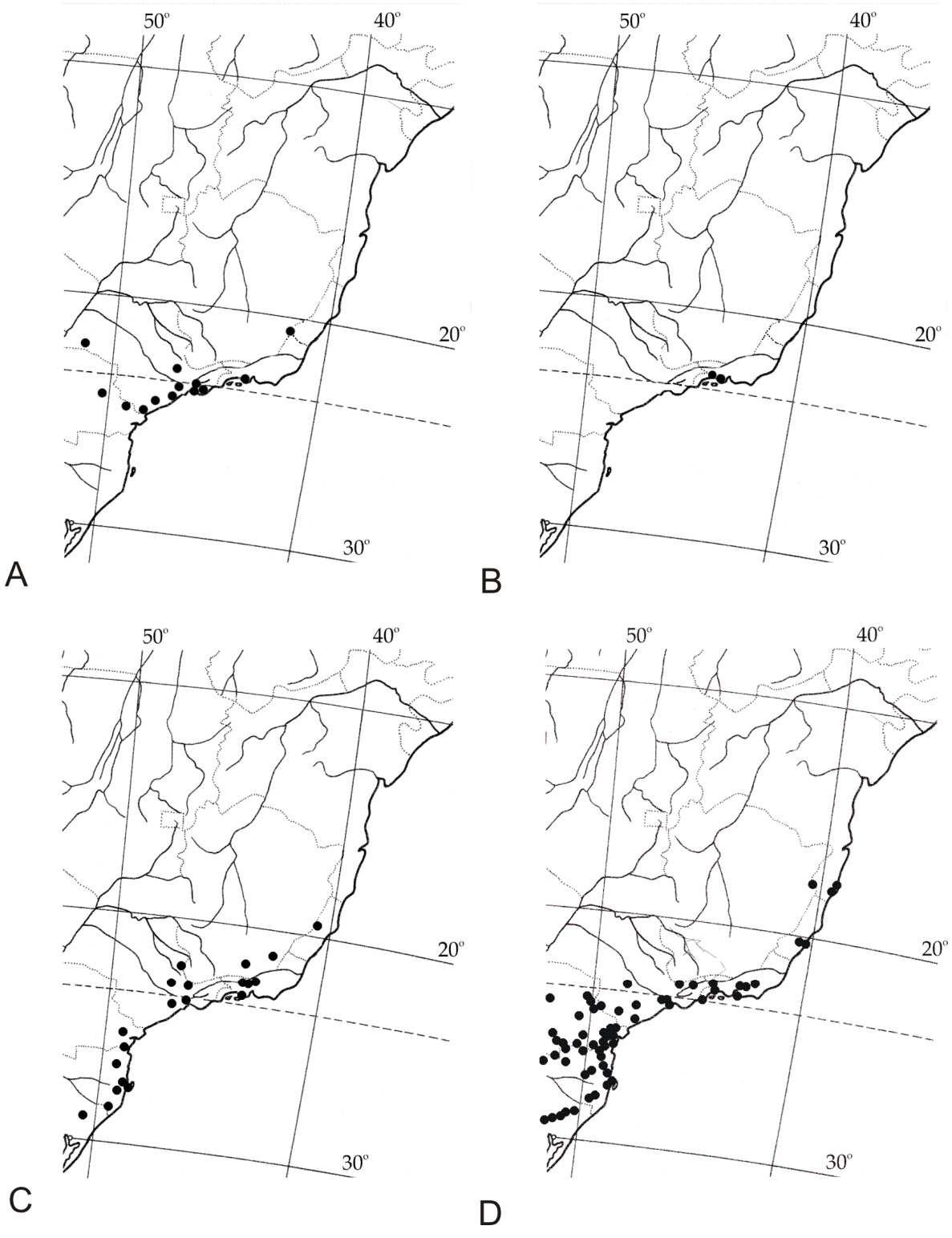


Fig. 9. Geographic distribution of species in south-southeastern Brazil. (A) *Maxillaria cepula.* (B) *M. echiniphyta.* (C) *M. ferdinandiana.* (D) *M. neowiedii*.

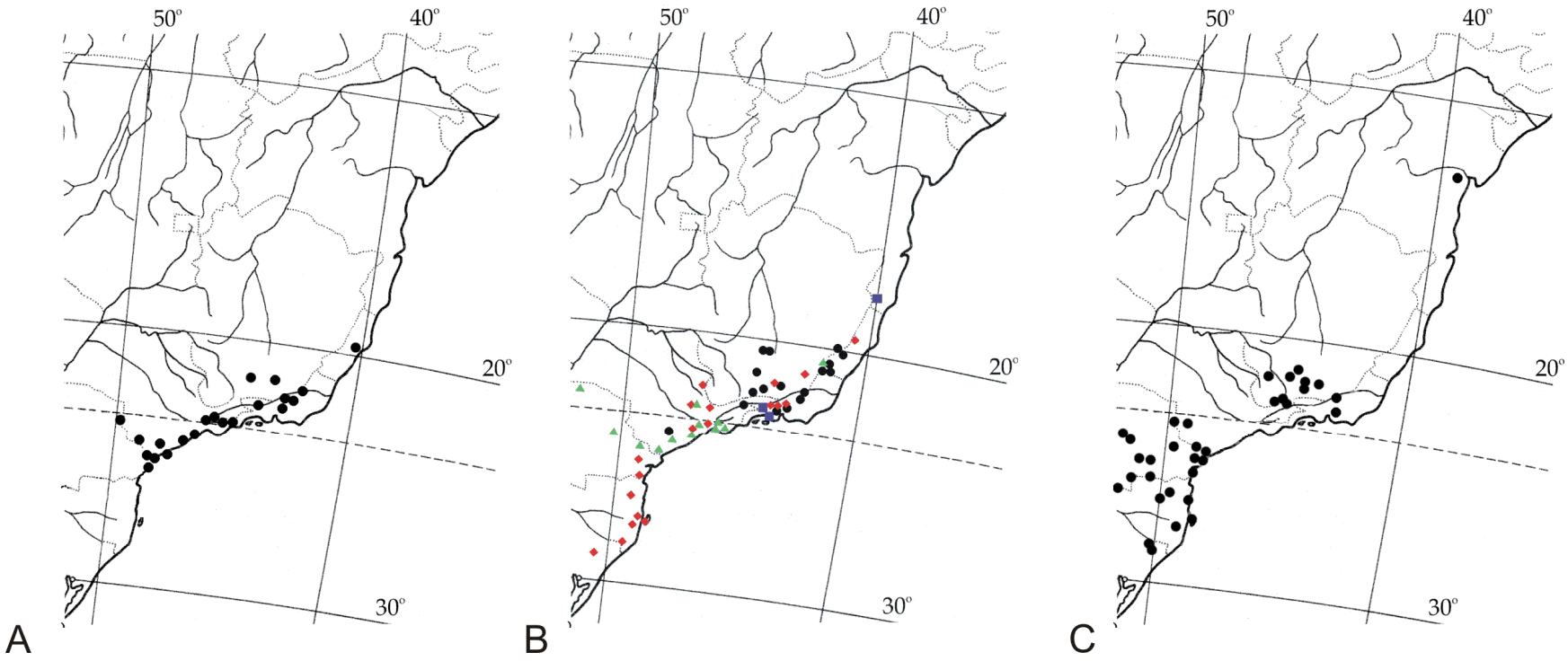


Fig. 10. Geographic distribution of species in south-southeastern Brazil. (A) *Maxillaria pumila.* (B) *M. subulata* var. *subulata* (green), *M. subulata* var. *angustipetala* (black), *M. subulata* var. *madida* (red), *M. subulata* var. *monophylla* (blue). (C) *M. vernicosa*.

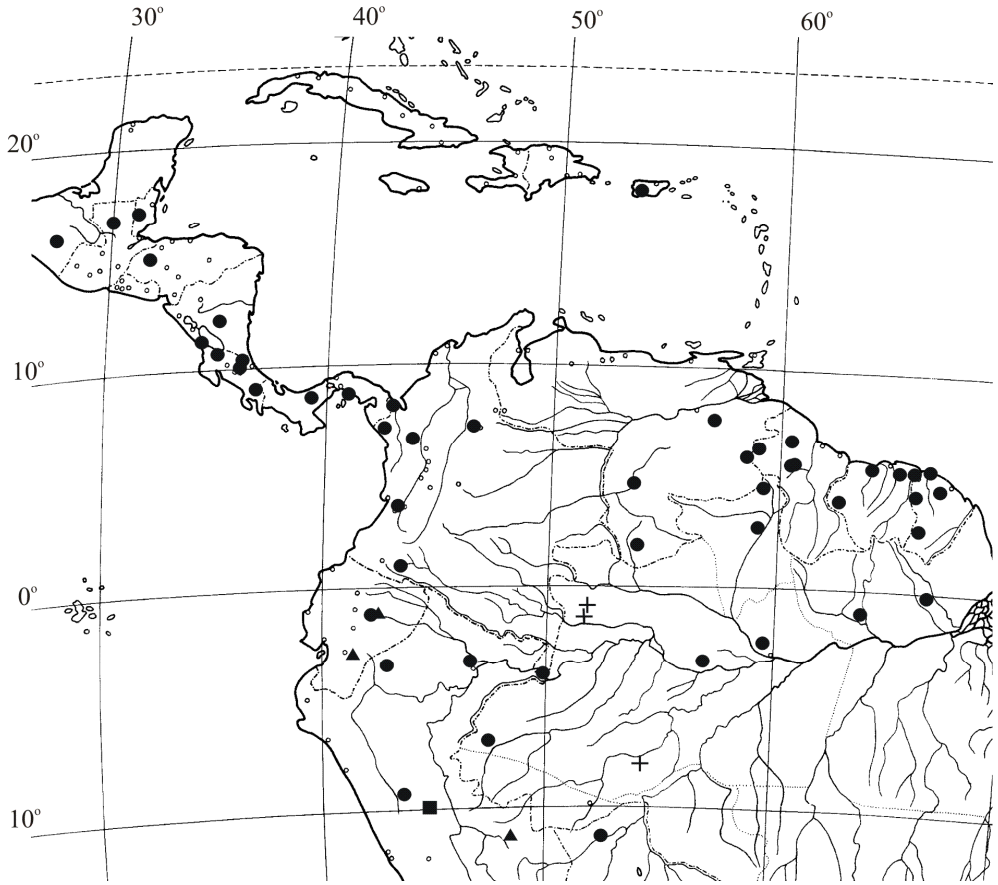


Fig. 11. Geographic distribution of species in northern-western South America and Central America. *Maxillaria nardoides* (triangules), *M. pacholskii* (square), *M. pumila* (crosses), *M. uncata* (circles).

References

Atwood JT. 1993 A revision of Maxillaria neglecta complex (Orchidaceae) in mesoamerica. *Lindleyana* 8: 25-31.

Barbosa Rodrigues J. 1877. Genera et Species Orchidearum Novarum, Vol 1 C. et H. Fleiuss, Sebastianópolis.

Barbosa Rodrigues, J. 1996. Iconographie des orchidées du Brésil, vol. 1 & 2. Sprunger, S., Cribb, P. & Toscano de Brito, A. (eds.) Basle: Friedrich Reinhardt Verlag.

Bennett DE, Christenson EA. 1993. Icones Orchidacearum Peruvianum: Plate 107. A. Pastorelli de Bennett, Lima.

Bennett DE, Christenson EA. 2001. Icones Orchidacearum Peruvianum: Plate 710. A. Pastorelli de Bennett, Lima.

Blanco MA, Whitten WM, Williams NH, Koehler S. 2006. Capillitial extrusion from fruits of *Maxillaria nardoides* (Orchidaceae: Maxillariinae). Orchids (Lindleyana) 75: 685-691.

Butzin F. 1978. In Berlin vorhandene Typen von Schlechters Orchideenarten [In Berlin existing types of Schlechter's orchid species]. Willdenowia 8: 401-407.

Butzin F. 1980. Typenstudien im Berliner Orchideen-Herbar: Arten von Kränzlin und Mansfeld. [Type studies in the Berlin orchid herbarium: species of Kränzlin and Mansfeld]. Willdenowia 10: 147-149.

Butzin F. 1981. Typenstudien im Berliner Orchideen-Herbar: Diverse markierte Typen [Type studies in the Berlin orchid herbarium: miscellaneous marked types]. Willdenowia 11: 119-121.

Butzin, F. 1983. Notiz ueber deutsche Orchideen im Herbar des Botanischen Museums Berlin-Dahlem [Notes on German orchids in the herbarium of the Botanical Museum Berlin-Dahlem]. Willdenowia 13: 393-396.

Cogniaux A. 1904. Orchidaceae III. Maxillarinae. In: von Martius CFP, Eichler AG, Urban I, eds. *Flora Brasiliensis.* Vol. 3(6). Lipsiae: Frid. Fleischer, 1–78.

Christenson E. 2003. Two Maxillarias, one old and one new. Orchid Review 111 (1253): 287-289.

Davies KL, Turner MP. 2004. Morphology of floral papillae in *Maxillaria* Ruiz & Pav. (Orchidaceae). Annals of the Missouri Botanical Garden 93: 75-86.

Dodson CH. 2002. Native Ecuadorian Orchids *Lepanthopsis – Oliveriana* 3: 433—651.

Dressler RL. 1993. Phylogeny and classification of the orchid family. Dioscorides Press, Portland.

Hoehne F.C. 1936. Orchidáceas dos herbários. Boletim do Museu Nacional 12(2): 36.

Hoehne FC. 1953. Orchidaceas. In: Flora Brasilica 10 (12, 7). Secretaria da Agricultura, São Paulo, pp. 223-341

Klinge J. 1898. Diagnoses Orchidearum Novarum in Calidariis, Horti Imperialis Botanici, Petropolitani Cultarum. Acta Horti Petropolitani 17 (1, 2): 10.

Mori SA, Ferreira FC. 1987. A distinguished Brazilian botanist, João Barbosa Rodrigues (1842-1909). Brittonia 39: 73-85.

Pabst GFJ, Dungs F. 1977. Orchidaceae brasiliensis II. Brücke–Verlag Kurt Schmersow, Hildesheim.

Vásquez RC, Dodson CH. 1982. Icones Plantarum Tropicarum. Orchids of Bolivia 6 (501—600): plate 554. Marie Selby Botanical Gardens, Sarasota.

Acknowledgements

We are much indebted to all institutions which provided specimens/information for study as well for all the help of their staff: Instituto de Botânica de São Paulo (E. Catharino, F. de Barros, F. Pinheiro, R. Peres and H. Michelan), Escola Superior de Agricultura "Luiz de Queiroz" (E. Ann–Veasey, G. Oliveira, J. Pontes) and the herbaria BM, BR, F, HB, K, MO, NY, OUPR, P, R, S, RB, SP, UEC, US, W. We sincerely thank A. Midori and R. Lourenço for the line drawings, and A. Martins, C. Drinkell, D. Roberts, E. Christenson, E. Pansarin, G. Romero, E. Smidt, J. Aranha, J. Semir, L. Kollman, M. Blanco, M. Whitten, N. Williams, R. Custódio, R. Gonçalves, V. Bittrich and W. Forster for all the help in developing this study. This work was supported by funding from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to S. Koehler (02/02161–7), M. C. E. Amaral (02/02162–3) and by a Prance Fellowship in Neotropical Botany from the Royal Botanical Gardens Kew to S. Koehler.

CONCLUSÃO GERAL

Esta tese teve como tema central a delimitação de espécies em um complexo de orquídeas neotropicais. Sem dúvida, a questão de quantas espécies devemos reconhecer em um determinado grupo tem intrigado taxonomistas por muito tempo. Entretanto, embora esta seja uma pergunta antiga, as ferramentas para estudar a formação e isolamento de espécies tornam-se cada vez mais numerosas e refinadas, permitindo uma abordagem múltipla de um problema originalmente restrito e que se situa no limite entre a taxonomia e a genética de populações.

O primeiro estudo desta tese teve como objetivo esclarecer limites de espécies e propor uma classificação mais estável para o complexo '*Maxillaria madida*'. Para resolver este problema foi desenvolvido um estudo filogenético baseado na variação em seqüências de DNA complementados por caracteres morfológicos e citogenéticos (contagem cromossômica e padrões de distribuição de heterocromatina). Os resultados corroboram o reconhecimento de seis espécies para este grupo, bem como sugerem que o clado '*M. pumila*' deva ser reconhecido como uma única espécie. Por utro lado, relações filogenéticas dentro dos sub-clados '*M. acicularis – M. madida*' e '*M. ferdinandiana – M. neowiedii*' foram inconclusivas e demandam estudos adicionais. Um destes estudos é apresentado no seguundo capítulo.

O capítulo 2 propôs-se a investigar o status taxonômico do sub-clado '*M. acicularis – M. madida*', que inclui as espécies *M. acicularis* e *M. madida* (incluindo *M. mosenii*), morfologicamente muito similares. Nosso objetivo foi reconhecer unidades taxonômicas que estivessem de acordo com padrões de variabilidade genética e morfológica encontrada. Para isso foi conduzido um estudo comparativo baseado em marcadores AFLP, considerando 56 populações, incluindo toda a variação morfológica encontrada no grupo. Os resultados não concordam com o reconhecimento de duas espécies como atualmente aceito, uma vez que foi detectada pouca diferenciação genética entre populações. Considerando a baixa diferenciação genética entre grupos morfológicos, juntamente com informações sobre ocorrência ecológica e distribuição geográfica, é proposto o reconhecimento de uma única espécie, *M. subulata,* subdividida em quatro variedades.

O reconhecimento formal das espécies do complexo '*Maxillaria madida*', incluindo o de uma única espécie para o sub-clado '*M. acicularis* – *M. madida*' e suas variedades é apresentada no capítulo seguinte (capítulo 3). Este capítulo aborda a revisão taxonômica para todo o grupo, incluindo também os táxons estudados no capítulo 1. Foram reconhecidas dez espécies: *M. cepula, M. echiniphyta, M. ferdinandiana, M. nardoides, M. neowiedii, M. pacholskii, M. pumila, M. subulata* (com quatro variedades propostas), *M. uncata* e *M. vernicosa*. Chaves de identificação, caracteres diagnósticos, breves descrições, listas de sinonímias taxonômicas, ilustrações e mapas de distribuição são apresentados.

Os estudos desenvolvidos nesta tese responderam algumas das perguntas inicialmente existentes para este grupo de orquídeas, mas também (felizmente) criam outras mais. Entender os processos responsáveis pelo surgimento de novas espécies é, sem dúvida, um tema fascinante da biologia. Complexos de espécies são particularmente interessantes porque constituem laboratórios vivos para o estudo de processos de especiação. Em um mundo onde a natureza é cada vez menor e as perguntas cada vez mais numerosas, a vida grita, com urgência, pela descoberta.

ANEXOS

Anexo 1. Protocolo utilizado no presente estudo para extração e precipitação de DNA (modificado a partir de Doyle e Doyle 1987)

Reagentes

- CTAB
- beta-mercaptoethanol
- clorofórmio/álcool isoamílico 24:1
- 3 M acetato de sódio, pH 4.8
- isopropanol 100%
- etanol 70%
- tampão Tris-EDTA (TE), pH 8.5
- microcentrífuga
- tubos eppendorf 1.5 ml
- pipetas plásticas 1-3 ml descartáveis
- gral e pistilo
- placa aquecedora, pré-aquecida à 65º C.

Extração

- Colocar 50-100mg de tecido no recipiente próprio para macerar (cuidado! tecido em excesso reduz a quantidade de DNA extraído). Adicionar 1.2 ml de solução de CTAB e 8µl de mercaptoetanol. Macerar tudo até a completa homogeinização da solução
- Transferir 1ml da solução para o tubo eppendorf
- Incubar amostras a 65 ºC por 20 minutos. No caso de amostras difíceis, deixar aquecendo por várias horas ou até o dia seguinte. Misturar as amostras ocasionalmente com auxílio de vórtice ou, para amostras mais delicadas, com uma pipeta automática
- Adicionar 500µl de clorofórmio/álcool isoamílico (24:1) - se a ponteira encostar no tubo, troque-a. Misturar em vórtice rapidamente até que seja obtida uma da solução leitosa
- Centrifugar por 5 minutos a 10.000 r.p.m. para separar fases - o clorofórmio ficará abaixo e a fase aquosa, contendo o DNA, acima
- Pipetar 750µl da solução aquosa e transferi-la para um novo tubo. Evitar aspirar a camada contendo clorofórmio. Caso ocorra a mistura das camadas, centrifugar novamente. Descartar o clorofórmio em um recipiente apropriado

Precipitação

- Adicionar acetato de sódio 3M à fase aquosa, de acordo com a fórmula:

vol. de acetato de sódio em µl =

vol. da fase aquosa em µl X 0.04 (750µl X 0.04 = 30µl)

- Adicionar isopropanol 100% de acordo com a fórmula:

isopropanol em µl = vol. fase aquosa X 0.65 (= 780 X 0.65 = ca. 510µl)

- Inverter os tubos manualmente. Ocasionalmente você poderá ver o DNA precipitando, mas não se preocupe se não conseguir ver nada
- Coloque tubos no freezer (-20C) por 3-4 horas ou preferencialmente até o dia seguinte

Purificação

- Centrifugar amostras na velocidade máxima (13 mil r.p.m.) por 20 min. Após a centrifugação um pellet deverá estar visível. Caso contrário, deixe precipitar por mais um dia e repita o procedimento
- Descartar cuidadosamente o sobrenadante
- Adicionar 1ml de etanol 70%. Inverta o tubo até que o pellet se destaque (alguns pellets simplesmente não saem do lugar!). Descarte o sobrenadante e repita o procedimento Pressione tubos em papel absorvente e coloque-os para secar a 60^{o}C por cerca de 20 min. O etanol deve ser eliminado completamente dos tubos
- Adicionar 75µl de tampão TE de acordo com a fórmula apresentada neste protocolo, que contém baixa concentração de EDTA – que pode interferir na reação de amplificação. Armazene amostras a curto prazo a -4 ^{o}C ou, a longo prazo, a -20 ^{o}C
- Correr um gel de agarose a 1% com a solução de DNA extraído para checar qualidade e a concentração do DNA

Anexo 2. Protocolos para as reações de amplificação para seqüenciamento de DNA[12]

i. Região ITS 1-2

Reagentes	**Vol./amostra (**µl)
Betaína	12
Tampão (10X)	5
$MgCl_2$ 25mM	7
DNTP Mix 2,5mM	1
Iniciador 3´- 5´	1
Iniciador 5´- 3´	1
ddH_2O	22,5

Programa para amplificação:

99 °C	10 min	
80 °C	adicionar *Taq*	
94 °C	2 min	
94 °C	45 s	X 30
60 °C	45 s	
72 °C	1 min	
72 °C	3 min	
4 °C	∞	

[12] Todas as reações de amplificação utilizaram 50-300 ng/µl de DNA, 0,5-1 unidade de *Taq*, primers com concentração de 10 pmol/µl, $MgCl_2$ com 3-4,5 mM conc. final e DNTPs com 2.5mM conc. inicial para cada dinucleotídeo e conc. final de 0,2 mM.

ii. Região *trnL-F*

Reagentes	**Vol./amostra (**µl)
Tampão (10X)	5
$MgCl_2$ 25mM	8
DNTP Mix 2,5mM	1
Iniciador 3´- 5´	1
Iniciador 5´- 3´	1
ddH_2O	33

Programa para amplificação:

94 °C	3 min	
94 °C	30 s	X 32
32 °C	30 s	
72 °C	75 s	
72 °C	5 min	
4 °C	∞	

iii. Região *matK*

Reagentes	**Vol./amostra (**µl)
Tampão (10X)	5
$MgCl_2$ 25mM	8
DNTP Mix 2,5mM	1
Iniciador 3´- 5´	1
Iniciador 5´- 3´	1
ddH_2O	33

Programa para amplificação:

94 °C	2 min		
94 °C	45 s	}	X 33
60 °C	45 s		
72 °C	2 min		
72 °C	4 min		
4 °C	∞		

iv. Região *atpB-rbc*L

Reagentes	**Vol./amostra (**µl)
Tampão (10X)	5
$MgCl_2$ 25mM	9
DNTP Mix 2,5mM	1
Iniciador 3´- 5´	1
Iniciador 5´- 3´	1
ddH_2O	33

Programa para amplificação:

94 °C	2 min		
94 °C	45 s	}	X 33
60 °C	45 s		
72 °C	2 min		
72 °C	3 min		
4 °C	∞		

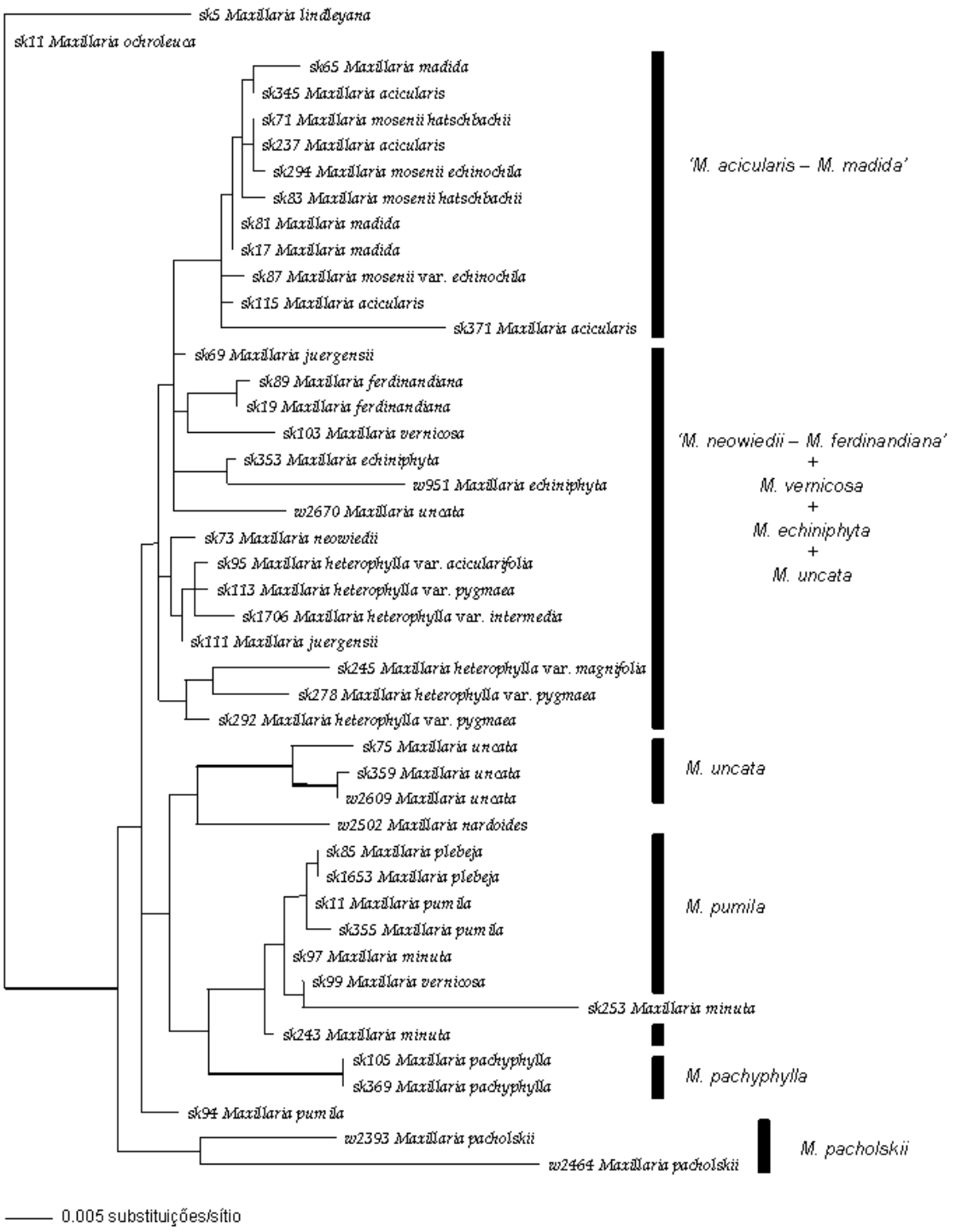
Anexo 3. Resultados das análises filogenéticas individuais das regiões de cloroplasto sequenciadas

Estatística de análises filogenéticas baseadas em regiões do cloroplasto considerando o critério de parcimônia máxima. Cada região foi analisada separadamente. [APM= número de árvores de parcimônia máxima obtido; C= comprimento das APM; IC= índice de consistência; IR= índice de retenção].

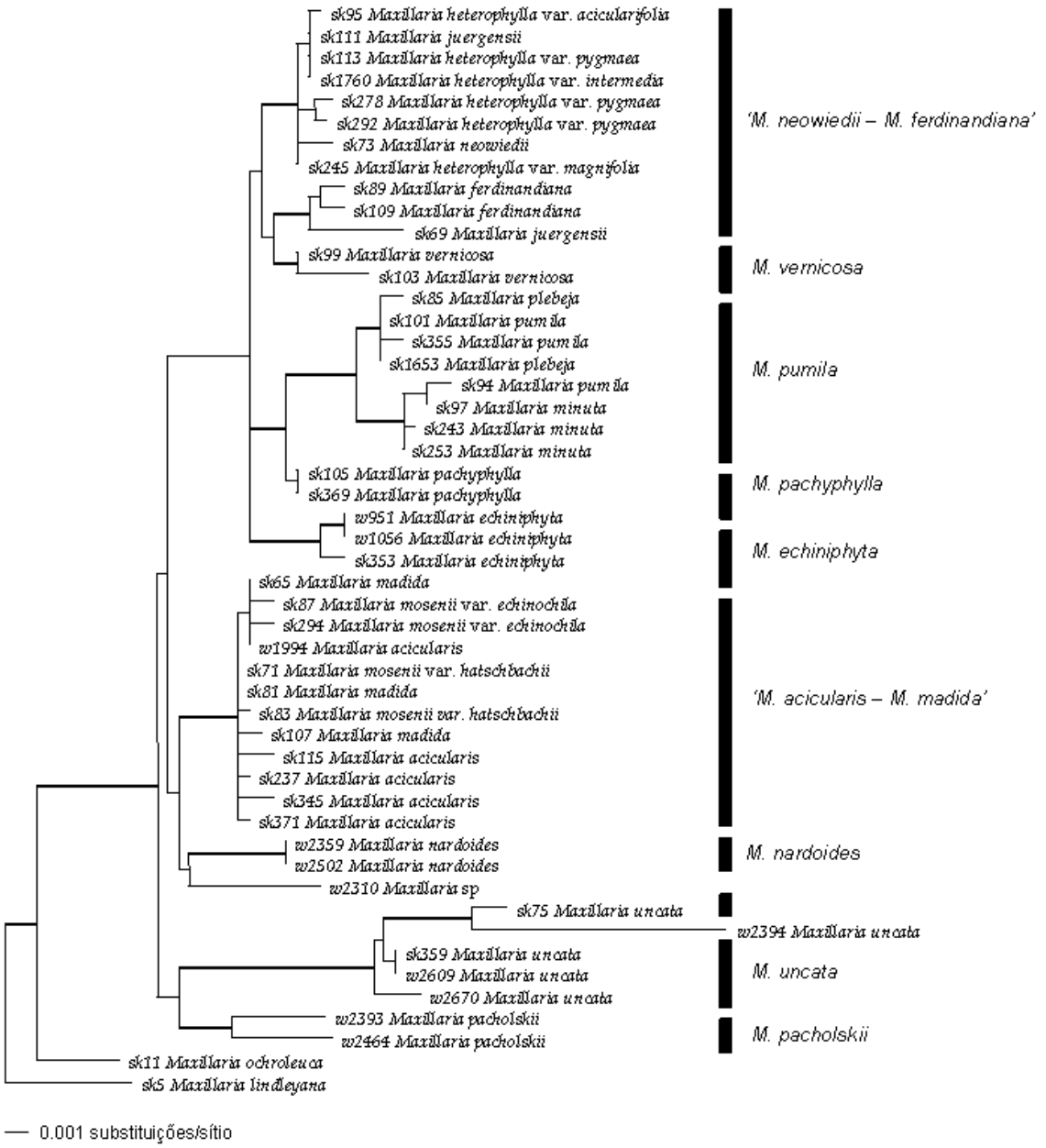
Conjunto de dados	# Táxons do grupo interno	Teste de chi-quadrado de homogeneidade	Estatística g1	No total de caracteres (informativos)	APM	C	IC	IR
atpB-rbcL	43	71.67 (df=126) p=0.99	-0,61	75 (6,1%)	25672	148	0,58	0,72
matK-trnK íntron	48	25.01 (df=141) p=1.0	-0,45	90 (7,4%)	327	136	0,73	0,93
trnL-trnF	37	279.11 (df=108) p=0	-0,82	111 (6,2%)	18163	147	0,81	0,95

Estatística de análises filogenéticas baseadas regiões do cloroplasto considerando o critério de verossimilhança máxima. Cada região foi analisada separadamente.

Conjunto de dados	# Táxons do grupo interno	Modelo selecionado	Freqüências de nucleotídeos estimadas	Valor-α da distribuição de probabilidades γ da taxa de variação entre sítios	Pinvar	-lnL value
atpB-rbcL	43	F81+G+I	A= 0.32 C= 0,11 G= 0,10 T= 0,46	1,03	0,56	3279.32
matK-trnK íntron	48	GTR+G	A= 0,31 C= 0,15 G= 0,15 T= 0,39	0,25	NA	3998.01
trnL-trnF	37	GTR+G+I	A= 0,35 C= 0,16 G= 0,15 T= 0,35	0,85	0,32	3065.33



Árvore de máxima verossimilhança resultante da análise de seqüências da região do espaçador *atpB-rbcL* do DNA de cloroplasto. Ramos com valores de *bootstrap* >75% estão marcados em negrito. Estatísticas referente às análise de verossimilhança máxima e parcimônia máxima são apresentadas nas tabelas acima.



Árvore de máxima verossimilhança resultante da análise de seqüências da região do gene *matK* e íntron *trnK* do DNA de cloroplasto. Ramos com valores de *bootstrap* >75% estão marcados em negrito. Estatísticas referente às análise de verossimilhança máxima e parcimônia máxima são apresentadas nas tabelas acima.

Árvore de máxima verossimilhança resultante da análise de seqüências da região *trnL-trnF* do DNA de cloroplasto. Ramos com valores de *bootstrap* >75% estão marcados em negrito. Estatísticas referente às análise de verossimilhança máxima e parcimônia máxima são apresentadas nas tabelas acima.

Anexo 4. Protocolos para as reações de amplificação para AFLP

i. Reação de Restrição-Ligação

1. São necessários 500ng de DNA diluídos em 5,5µl de ddH_2O
2. Antes de preparar a solução para reação, desnature o volume a ser utilizado dos adaptadores por 5 mim a 95ºC

Solução I

Reagentes	**Vol./amostra (µl)**
10X Tampão Ligase	0,1
0,5 M NaCl	0,1
1 mg/ml BSA	0,05
50 U/µl Mse1	0,02 (~1,0U)
80 U/µl EcoRI	0,06 (~5,0U)
20 U/µl Ligase	0,05 (~1,0U)
ddH_2O	completar até 1

Solução II

Reagentes	**Vol./amostra (µl)**
10X Tampão Ligase	1
0,5 M NaCl	1
1 mg/ml BSA	0,5
adaptador Mse 1	1
adaptador EcoRI	1

- ❖ Após o preparo das soluções I e II, centrifugue instantaneamente os tubos por 10 seg
- ❖ Misture a solução I com a II. Adicione 5,5µl em cada tubo contendo 5.5µl de DNA e centrifugue instantaneamente os tubos por 10 seg.
- ❖ Incube as amostras em um termociclador por 2h a 37ºC[13]

[13] É muito importante utilizar sempre o mesmo termociclador em todas as reações, uma vez que a técnica de AFLP é muito sensível a variações sutis de temperatura nas reações de amplificação.

- ❖ Ao término da reação, utilize 5,5µl para correr um gel de agarose 1,5%, de forma a verificar o sucesso da reação. Imediatamente depois adicione 95 µl de tampão TE 0,1M em cada tudo, misturando bem. Armazene as amostras a -20°C

ii. Reação de amplificação pré-seletiva

Reagentes	**Vol./amostra (µl)**
AFLP Core Mix	7,5
iniciadores EcoRI/Mse1	0,5

3. Adicone 8 µl em cada tubo mais 2 µl do produto da reação de R-L

Programa para amplificação:

72 °C	2 min	
94 °C	20 seg	X 20
56 °C	30 seg	
72 °C	2 min	
60 °C	30 min	
4 °C	∞	

4. Após o término da reação, corra um gel de agarose 1,5% com 5 µl do produto da reação
5. Dilua o volume restante em 95 µl de TE 0,1
6. Armazene as amostras a -20°C

iii. Reação de amplificação seletiva

Reagentes	**Vol./amostra (µl)**
AFLP Core Mix	7,5
iniciador EcoRI (Cxx)	0,5
iniciador Mse 1 (Axx)	0,5

7. Adicone 8,5 µl em cada tubo mais 2 µl do produto da reação de R-L

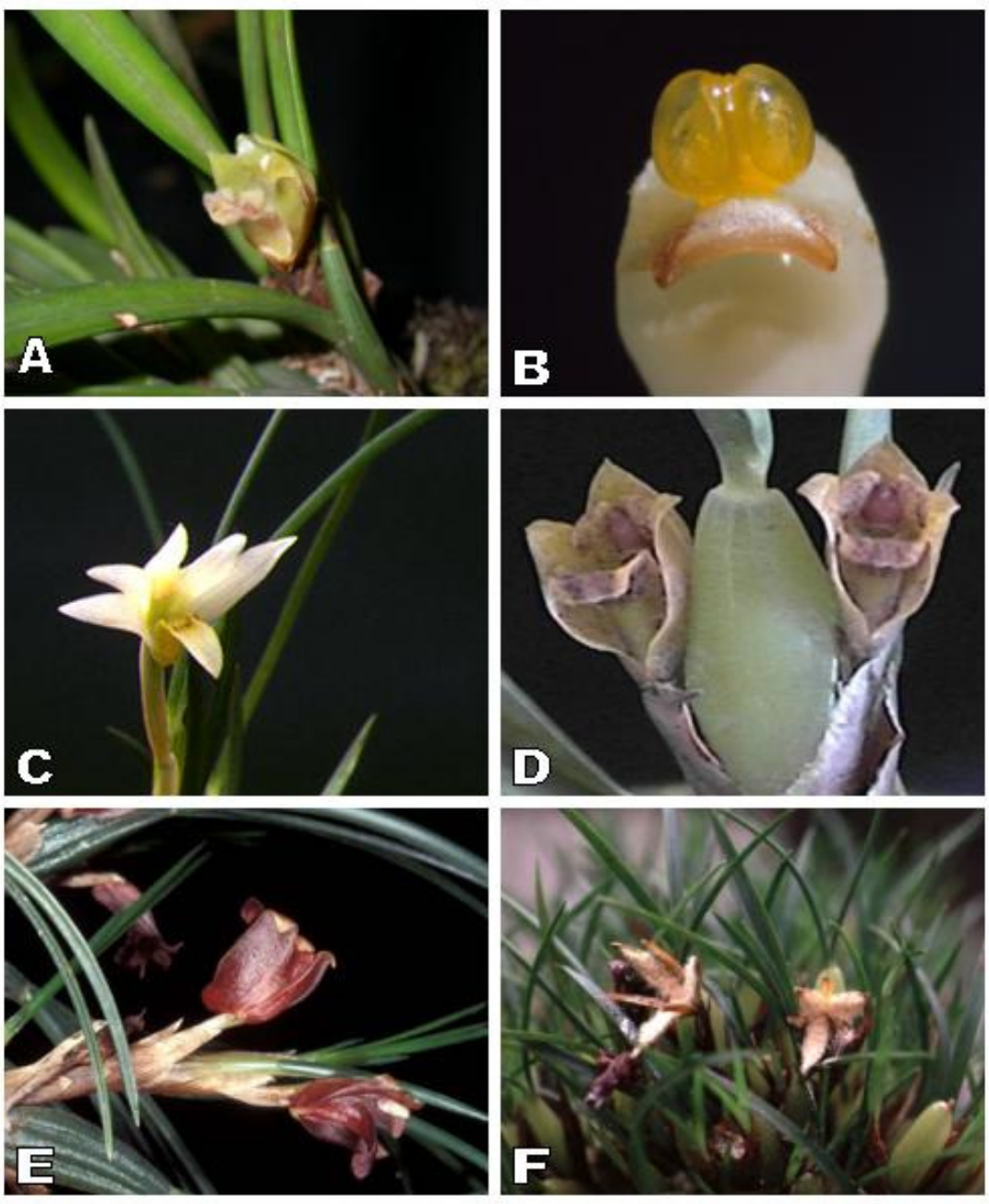
Programa para amplificação:

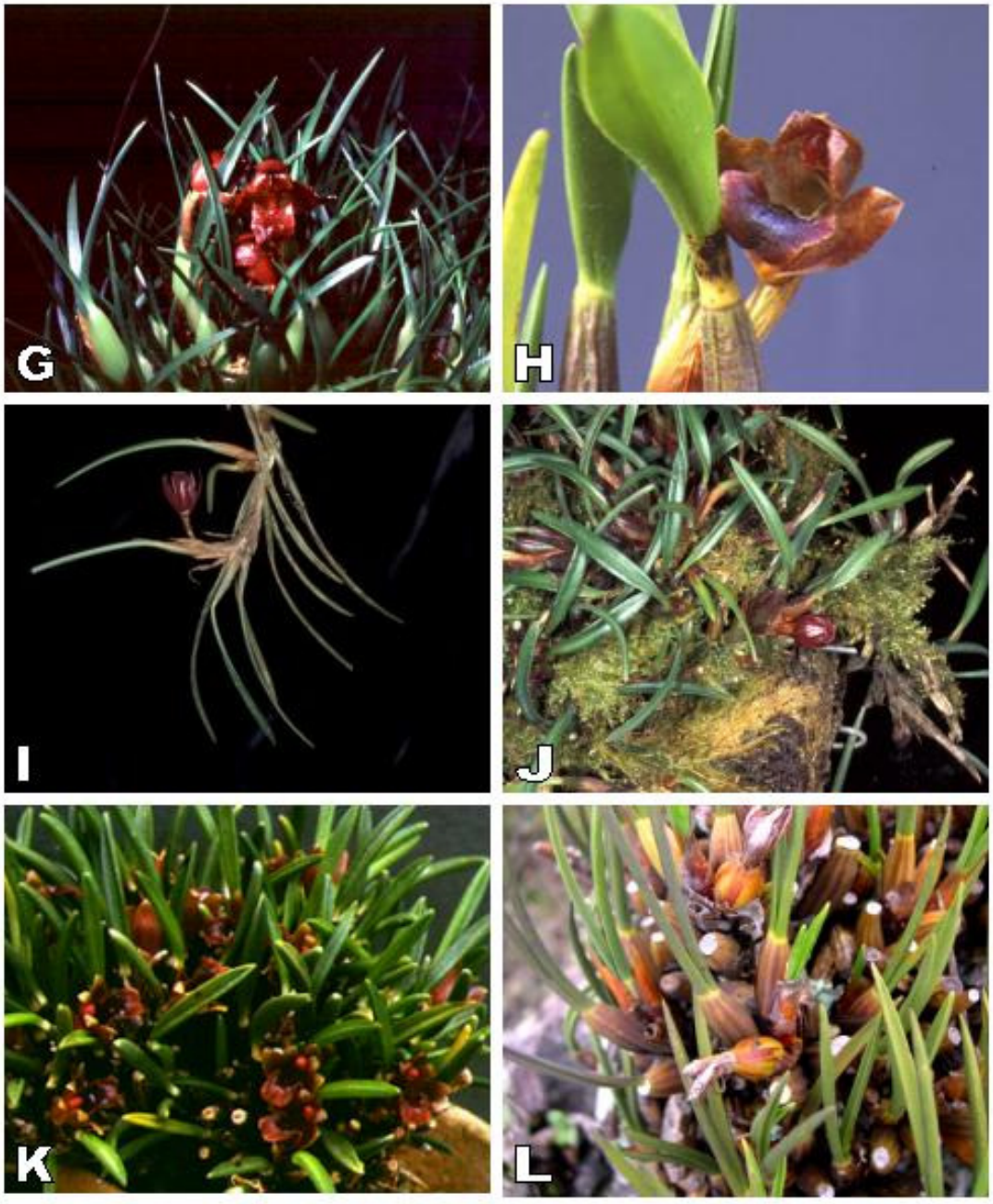
94 °C	2 min	
94 °C	20 seg	X 10 (diminuindo 1 °C/ciclo)
66 °C	30 seg	
72 °C	2 min	
94 °C	20 seg	X 20
56 °C	30 seg	
72 °C	2 min	
72 °C	2 min	
60 °C	30 min	
4 °C	∞	

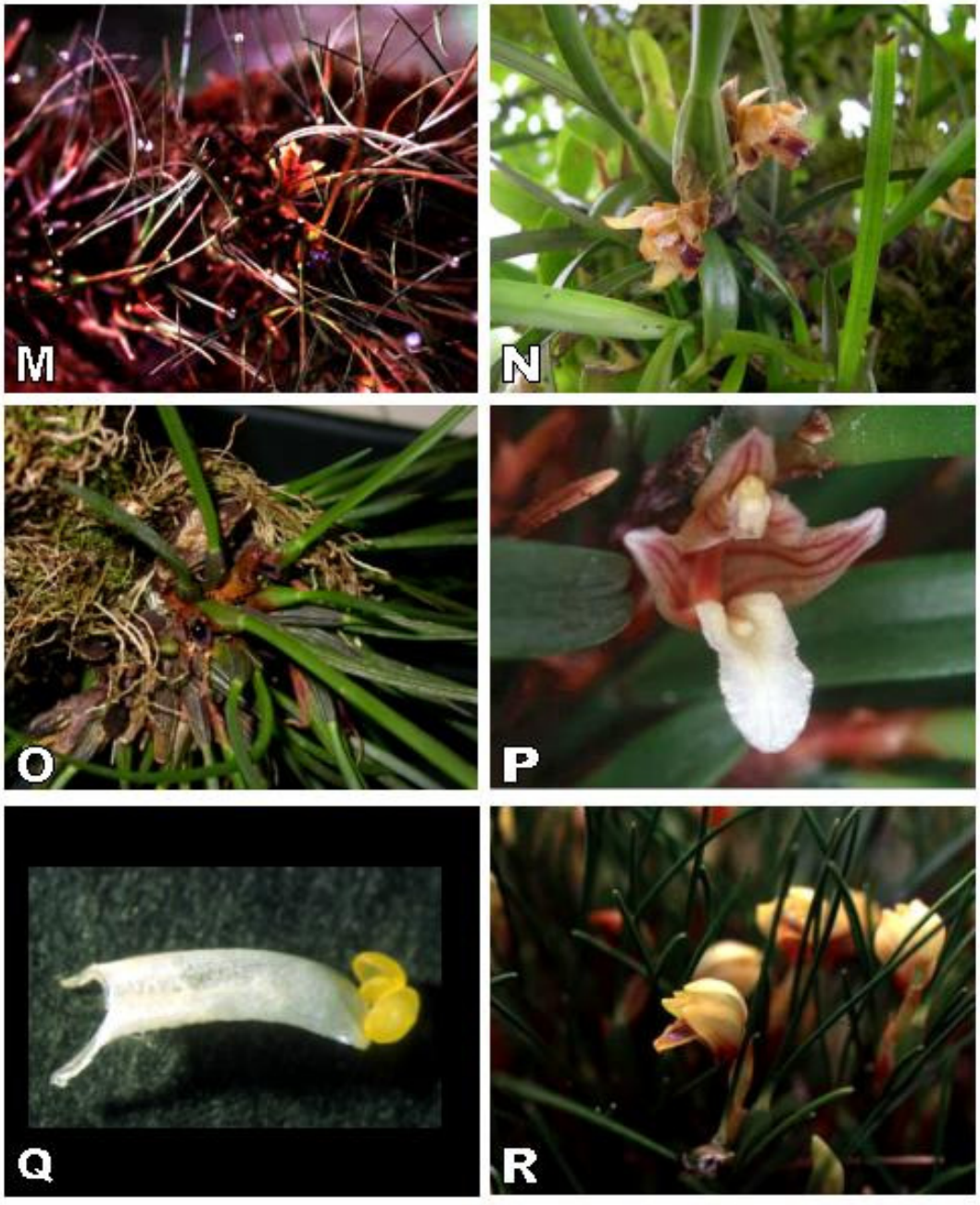
iii. Preparação de amostras para o seqüenciamento

8. Trabalhe sempre com as amostras em gelo
9. Prepare uma solução contendo 10µl de *high dye* e 0,2 de *Rox* (padrão) para cada amostra. Aliquote 10µl deste solução em cada tubo
10. Em cada tudo adicione também 1µl de cada combinação de iniciadores (NED, FAM, JOE)
11. Aqueça os tubos a 95 °C por 5 min e imediatamente após coloque-os em gelo por mais 5 min
12. As amostras estão prontas para serem colocadas no seqüenciador

Anexo 5. Fotos do táxons reconhecidos neste estudo. Espécies e variedades reconhecidas para o complexo *´Maxillaria madida´* de acordo com os resultados obtidos nesta tese. (A-B) *M. cepula* (=*M. pachyphylla*) [(B) detalhe do polinário no ápice da coluna]; (C) *M. echiniphyta;* (D) *M. ferdinandiana;* (E) *M. nardoides;* (F-H) *M. neowiedii* [(F) indivíduo com fruto]; (I) *M. pacholskii;* (J-K) *M. pumila;* (L) *M. subulata* var. *subulata* (=*M. mosenii* var. echinochila) [indivíduo com fruto]; (M) *M. subulata* var. *angustipetala* (= *M. acicularis*); (N) *M. subulata* var. *madida* (=*M. madida*); (O) *M. subulata* var. *monophylla* (*M. madida* var. *monophylla*); (P-Q) *M. uncata;* (R) *M. vernicosa*




G
H
I
J
K
L


M
N
O
P
Q
R