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Phylogeny of Macroptilium (Leguminosae): morphological, biochemical and molecular evidence

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Abstract

Macroptilium (Benth.) Urban (Phaseoleae, Papilionoideae, Leguminosae) is an American genus of legumes, belonging to subtribe Phaseolinae along with other economically important genera, such as *Vigna* Savi and *Phaseolus* L. (the common bean genus). Cladistic analyses based on morphological, biochemical (storage seed proteins) and molecular (nuclear and plastid DNA sequences) data were performed on the 18 species currently ascribed to the genus, exploring several character weighting strategies. Equal weights, implied weighting and different transversion/transition costs were applied. The three data sets were first analyzed with separate partitions, and then combined into a single matrix. This study is the first one to analyze all the species of the genus from a cladistic point of view. In all the most parsimonious trees obtained, *Macroptilium* is monophyletic with excellent support values. Two monophyletic clades are recovered in almost all the analyses. Both are compound by nine species, and they constitute two sections of *Macroptilium*. Several interspecific relationships inside the genus are discussed.

The genus *Macroptilium* (Benth.) Urban is included in subtribe Phaseolinae (Phaseoleae, Papilionoideae, Leguminosae), along with 20 other genera of legumes that occurred in a monophyletic group, according to recent molecular phylogenetic analyses (Lewis et al., 2005). Some of their species were first recognized by Bentham (1837, 1865) as a section of *Phaseolus* L. (the "common bean" genus) based on the observation that the petals that form the wings of the flowers are larger than the standard petal. Urban (1928) raised Bentham's section to the genus level, but his taxonomy was not followed until many years later, when authors like Hutchinson (1967), Maréchal (1970) and Verdcourt (1970) studied taxonomic aspects of the subtribe Phaseolinae. Since then, the status of *Macroptilium* as a genus has not been questioned. Most of the species currently assigned to *Macroptilium*, were described by Bentham (1837, 1865) as belonging to two sections of *Phaseolus*: *Macroptilium* Benth. and *Microcochle* Benth. Piper (1926) also mentioned these sections in his taxonomic review of the American Phaseolinae. Urban (1928) did not present any division of the genus in his work. Maréchal et al. (1978), based on a numerical analysis of morphological and biochemical characters, considered *Macroptilium* to be a very uniform genus that cannot be divided into sections. Lackey (1983) on the other hand, grouped the species assigned to *Macroptilium* into three sections: *Macroptilium, Microcochle* (Benth.) Lackey and *Monophyllum* Lackey. According to this last author, section *Macroptilium* is characterized by its large, pluriovulate flowers. Section *Microcochle* has smaller flowers, few ovules per flower and orbicular seeds. Finally, section *Monophyllum*, with only one species, is intermediate between the other two, and is distinguished by its unifoliolate leaf and lateral stigma (Lackey, 1983).

The 18 species presently assigned to *Macroptilium* are distributed from the southern United States to central Argentina; Chile is the only South American country where no *Macroptilium* species are found. The greatest diversity is in Mexico, where nine species have been described, and in middle South America, with 12

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Macroptilium taxa. Nine of these 12 species grow in Argentina, distributed from the northern to the central provinces.

Since Lackey's (1983) studies, no other surveys of the genus as a whole have been published, although regional treatments have appeared (Barbosa Fevereiro, 1986; Drewes, 1997), based mainly on morphological data.

With the major aim of proposing a phylogenetic classification of *Macroptilium*, and secondarily to analyze some interspecific relationships within the genus, a phylogenetic analysis of the 18 species of *Macroptilium* was performed using three different sources of characters: morphological, biochemical and molecular. Features of vegetative, floral and pollen morphology were scored. Electrophoretic data from seed storage proteins were used as biochemical characters, a data source that has proved to be very helpful in previous legume phylogenetic studies (Przybylska, 1995; Maquet et al., 1999; Burghardt, 2000a,b) to clarify species delimitations at both supra- and intraspecific levels (Espert and Burghardt, 2003; Sammour, 1994). DNA data from the *trnL–trnF* intergenic spacer (IGS) region of the chloroplast were collected, a region used successfully to resolve generic and species-level relationships in other legume groups (Brouat et al., 2001; Ainouche et al., 2003; among others). Sequences from the nuclear ribosomal DNA internal transcribed spacers (ITS) were analyzed as well. ITS has traditionally been the major nuclear region used in plant phylogenetic studies (Álvarez and Wendel, 2003). However, if concerted evolution fails to homogenize the possible ITS paralogues that may arise, the possibility of unknowingly sampling sequences with different evolutionary histories becomes a real danger to phylogenetic analyses (Baldwin et al., 1995). Because intraspecific variation of ITS sequences has been detected in several plant groups (Mayol and Rosselló, 2001; Bellarosa et al., 2005; Gottlieb et al., 2005), basic features of the sequences such as integrity of conserved motifs and thermodynamic stability of the secondary structures of the RNA transcripts should be carefully inspected in order to avoid phylogenetic incongruence.

Hypotheses of monophyly of the genus *Macroptilium* and of its sections have never before been tested, so different character weighting strategies were performed in order to evaluate topological stability of the hypotheses. The present work constitutes the first molecular exploration within *Macroptilium*.

Materials and methods

Taxon sampling

Fifty-one accessions from the 18 species of the genus *Macroptilium* were analyzed. Live material from three species, *M. pedatum* (Rose) Maréchal & Baudet from North America, *M. martii* (Benth.) Maréchal & Baudet and *M. monophyllum* (Benth.) Maréchal & Baudet from Brazil and Paraguay, were not available for the biochemical and molecular studies, therefore only morphological data from herbarium specimens were recorded for these taxa. The sampling also included five species related to *Macroptilium*, belonging to subtribe Phaseolinae (Delgado Salinas et al., 1999), which formed the outgroup. The list of all species included in this study, their origin, voucher identification numbers and herbaria where they are deposited, are given in Appendix 1.

Morphological data

Forty-four morphological characters were scored for all species of *Macroptilium* and the outgroup. The list of all morphological characters included and their states are shown in Appendix 2. Ecological features, external morphology, deep morphology and palynological data were studied. The characters were obtained from fresh plant material, herbarium specimens and from literature reports. All the plant material examined were deposited in the herbarium of the University of Buenos Aires, Argentina (BAFC, acronyms according to Holmgren et al., 1990). Herbarium specimens were borrowed from: Universidad Nacional de Salta, Argentina (MCNS); Jardin Botanique National de Belgique, Belgium (BR); Universidad Nacional de Asunción, Paraguay (FCQ); Conservatoire et Jardin botaniques de la Ville de Genève, Switzerland (G); Smithsonian Institution, USA (US); and Centro Internacional de Agricultura Tropical, Colombia (CIAT). The work of Delgado Salinas and Torres Colin (2004) were used to clarify the codification of some characters of *M. atropurpureum* (Sessé & Moc. ex DC.) Urb. and *M. ecuadoriensis* (Sessé & Moc. ex DC.) L. Torres-Colin & A. Delgado.

Biochemical data

Seed storage proteins were extracted by grinding one seed at a time, and mixing the powder with a 0.5 M NaCl solution. The suspension was centrifuged at 10 000 r.p.m. for 10 min and the supernatant was mixed with an equal volume of cracking buffer (0.125 M Tris–HCl pH 6.8, 4% w/v sodium dodecyl sulfate, 20% w/v glycerol, 10% w/v 2-mercaptoethanol, 0.001% w/v bromophenol blue) and boiled for 2 min. One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using the procedure of Laemmli (1970). Protein bands were visualized with a Coomassie Blue staining after the gels were run.

A data matrix was constructed using presence/absence of protein bands on the electrophoretic gels. A total of 140 polypeptide bands were scored. The matrix is presented in Appendix 3, where only the parsimony informative characters are shown.

Molecular data

Total genomic DNA were obtained from fresh leaves or silica gel-dried leaf material according to the CTAB extraction protocol modified from Milligan (1998). Two DNA regions were analyzed. The internal transcribed spacer regions of nuclear DNA (ITS-1 and ITS-2) were amplified by polymerase chain reaction (PCR) using primers ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), ITS-5 (5′-GGA AGG AGA AGT CGT AAC AAG G-3′) and the internal primers ITS-2 (5′-GCT GCG TTC TTC ATC G-3′) and ITS-3 (5′-TCG ATG AAG AAC GCA GC-3′), designed by White et al. (1990). Amplifications were carried out in a 50 μL reaction mixture containing 0.225 mM dNTPs, 10% PCR buffer, 0.8 mM $MgCl_2$, 5×10^{-7} mM of each primer, 0.5 units of *Taq* DNA polymerase (Invitrogen) and 30–80 ng of template DNA. Reactions were performed in a thermocycler (Eppendorf Mastercycler) under the following conditions: an initial cycle of 94 °C for 4 min; 30 cycles at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min; followed by a final extension cycle of 7 min at 72 °C. The intergenic spacer between *trnL* and *trnF* genes of chloroplast DNA were amplified with primers e (5′-GGT TCA AGT CCC TCT ATC CC-3′) and f (5′-ATT TGA ACT GGT GAC ACG AG-3′), designed by Gielly and Taberlet (1994). A total of 50 μL of reaction mixture contained 0.200 mM dNTPs, 10% PCR buffer, 2.5 mM $MgCl_2$, 1 μM of each primer, 0.5 units of *Taq* DNA polymerase and 30–80 ng of template DNA. Amplifications were carried out in a thermocycler programmed for 31 cycles as follows: 2.5 min at 94 °C, 30 cycles of 1 min at 94 °C, 45 s at 52 °C and 1 min at 72 °C, followed by one final extension cycle of 4 min at 72 °C.

All PCR reactions were monitored by inclusion of a negative control. Amplification products were size separated by electrophoresis on 2.5% agarose gels in 1 × TAE buffer, stained with ethidium bromide and visualized under ultraviolet light. Bands were excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen).

Automated sequencing was performed at the DNA Sequencing Facilities of the Universidad de Alcalá (Spain) and Macrogen Inc. (South Korea), with the same primers used in the amplification step. The boundaries of the ITS and IGS regions were determined by comparison with known sequences of related species. All new sequences have been deposited in GenBank under accession numbers DQ888767–DQ888800. The sequences of *M. atropurpureum* (AF115138), *M. ecuadoriensis* (AY508736), *M. erythroloma* (Mart. ex Benth.) Urb. (AF069117), *M. gracile* (Poepp. ex Benth.) Urb. (AY508739), and outgroup species *Vigna adenantha* (G. Mey) Maréchal, Mascherpa & Stainier (AF069119), *Dolichopsis paraguariensis* Hassler (AF069116), *Phaseolus augusti* Harms (AF115179), *P. lunatus* L. (AF115175) and *P. vulgaris* L. (AF115166) were taken from GenBank and included in the analysis.

The possible existence of paralogous sequences for the ITS-1 and ITS-2 regions were checked by analyzing the length of the sequences, the G + C content, the presence and length of conserved domains, secondary structures and free energy, as suggested by Mayol and Rosselló (2001). Fold predictions were made at the Quikfold web server (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form3.cgi) by use of the MFOLD program version 3.1 (Zuker, 2003). Foldings were conducted at 37 °C by use of a search within 5% of the thermodynamic optimality set.

Sequence alignments were made with the DIALIGN program (Morgenstern et al., 1998), using a threshold value of 10.

Phylogenetic analyses

Prior to the cladistic searches, parsimony uninformative characters were deactivated. Morphological, biochemical and molecular data sets were first analyzed separately and then simultaneously using the T.N.T. program ver. 1.0 (Goloboff et al., 2003), with different weighting strategies. Equal weights and implied weights were applied to morphological, biochemical and to the combined data matrices. Equal weights, implied weights and differential transversion/transition transformation costs were applied to the molecular data set. All characters were treated as non-additive. Preceding the combined analysis, the congruence among data sets was measured by the ''Incongruence Length Difference'' test (Farris et al., 1995) using the WinClada program ver. 1.00.08 (Nixon, 2002).

Analyses were conducted using a heuristic tree searching procedure: 20 random addition sequences plus TBR, retaining 10 cladograms per replicate, keeping up to 10 000 trees.

The biochemical matrix was analyzed with the exact search algorithm implemented in T.N.T. (''branch-and-bound'') due to its small number of taxa. In some analyses, constrains of monophyly of internal *Macroptilium* clades were applied. This was achieved in order to test how much the optimal length differed with the length obtained when no constrains were used. Synapomorphies were mapped also using T.N.T. ver. 1.0 (Goloboff et al., 2003), on the strict consensus trees of the combined analyses.

Bremer supports (Bremer, 1994) were calculated finding up to 10 extra steps suboptimal trees, retaining 10 000 trees in the memory buffer. Jackknife values (JK) (Farris et al., 1996) were found resampling the matrix 1000 times, with a 36 removal probability.

Results

Morphological data

The data matrix of 23 species and 44 morphological characters (Appendix 2) was first analyzed under equal weights. The search resulted in 26 most parsimonious trees (MPT) of 100 steps, Consistency Index (CI) 0.52 and Retention Index (RI) of 0.796; the strict consensus tree of these cladograms is shown in Fig. 1. In this tree, the genus is monophyletic and two main clades are observed. One clade has a 51% JK and a Bremer support (BS) of 1, and it comprises eight species, most of them traditionally assigned to section *Macroptilium*. This group of species plus *Macroptilium panduratum* (Benth.) Maréchal & Baudet, that is also assigned to the section, will be identified as "Clade A" from now on. The second clade has nine species, and we named it "Clade B" (81% JK, 3 BS). This group is composed mostly of species described in section *Microcochle*. The position of *M. panduratum* is not resolved in the consensus tree; in 12 of the 26 most parsimonious cladograms, the species is found basal to "Clade B", and in the remaining 13 MPT this taxon occurs basal to "Clade A".

The implied weighting search strategy produced a single MPT of the same topology under concavity constant (K) from 1 to 6. This cladogram is identical to one of the MPT obtained under equal weights. In this tree, genus *Macroptilium* is monophyletic, with high support values (89% JK) and is composed of two groups, that correspond to "Clade A" and "Clade B" (tree not shown).

Biochemical data

The analysis of the seed storage protein matrix of 19 taxa and 40 parsimony informative characters resulted in 42 MPT, 60 steps long (CI = 0.66 and RI = 0.78) when an exact (branch and bound) search was performed. In the strict consensus tree, *Macroptilium* is monophyletic, but the internal relationships are poorly resolved, as most remain as a polytomy (tree not shown). Under implied weights, a single MPT was obtained with all concavity constants (K = 1–6), showing a monophyletic *Macroptilium* composed of two clades (Fig. 2). One of these groups contains seven of the nine species of "Clade B" (observed in the morphological analysis). The other one consists of species of "Clade A" and *M. sabaraense* (Hoehne) V.P. Barbosa ex G.P. Lewis, although this last clade has very low support values (< 50% JK, Fig. 2).

Molecular data

Prior to the analyses, the nuclear ITS sequences were examined for orthology. The sequence length and G + C content (above 50%) were those expected for angiosperms (Baldwin et al., 1995). All of the sequences conformed to thermodynamically stable secondary structures and contained the expected conserved domains (Mayol and Rosselló, 2001). We therefore concluded that none of the sequences obtained here are paralogous and they appear to be functional regions of the nuclear genome. This detailed inspection allows us to include these data in the analyses with greater

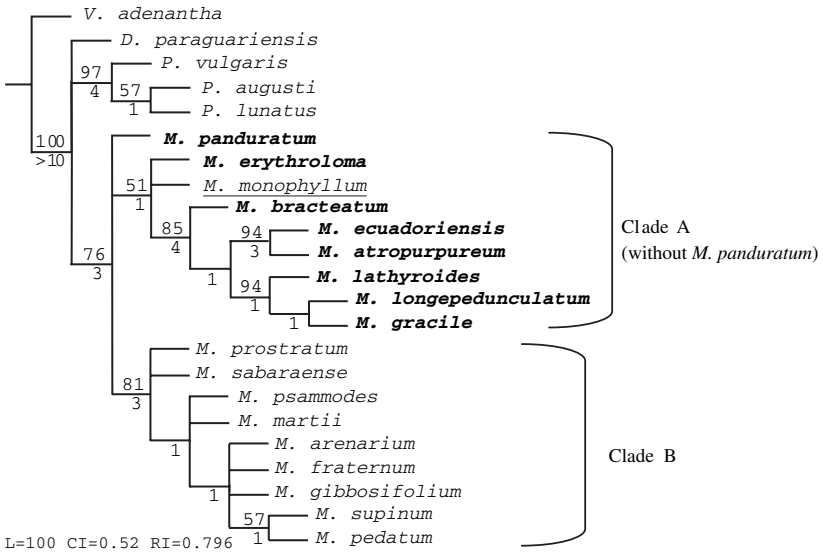

Fig. 1. Strict consensus of 26 MPT of the morphological analysis under equal weights. Jackknife values over 50% and Bremer support values are shown above and below branches, respectively. Length (L), Consistency Index (CI) and Retention Index (RI) of the MPT are indicated. Lackey's sectional delimitation is shown in italics (sect. *Microcochle*), bold (sect. *Macroptilium*) and underlined (sect. *Monophyllum*). Identification of "Clade A" and "Clade B" is explained in the text.

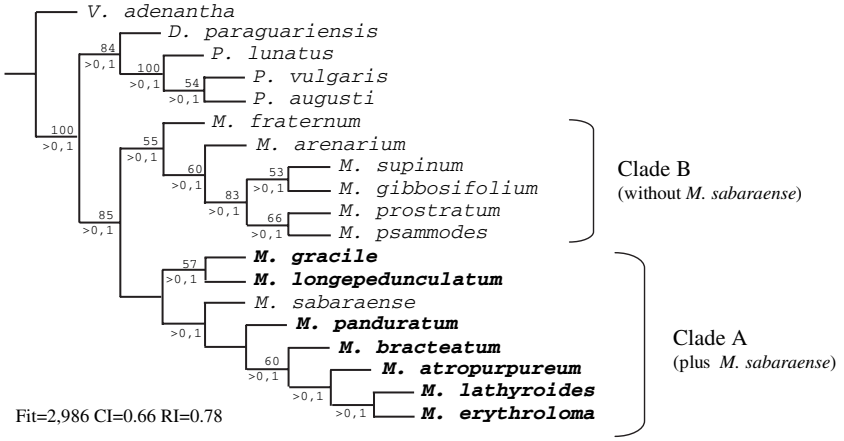

Fig. 2. Cladogram obtained under implied weighting (K = 5) of the biochemical matrix. Jackknife values over 50% and Bremer support values are shown above and below branches, respectively. Lackey's sectional delimitation is shown in italics (sect. *Microcochle*) and bold (sect. *Macroptilium*). Fit (F), Consistency Index (CI) and Retention Index (RI) are indicated.

confidence, as we agree with Gottlieb et al. (2005) in that phylogeneticists should always be careful when using ITS sequences in phylogenetic studies.

A matrix of 20 taxa and 576 nuclear DNA characters, 179 of them parsimony informative, was obtained after the alignment. A second matrix with 13 taxa and 428 characters (17 of them parsimony informative) was recovered from the *trnL–trnF* plastid region. Both molecular data sets were combined into a single matrix for analysis. In all the searches, the genus *Macroptilium* was monophyletic with high support values (99% JK). When the molecular matrix was submitted to a search under equal weights, two MPT were obtained, each 498 steps long (CI = 0.58 and RI = 0.721). These differed only in the position of *M. fraternum* (Piper) Juarez & S. Pérez inside "Clade B". The strict consensus of these two cladograms is shown in Fig. 3.

For all sets of transition/transversion costs (2/1, 4/1, 8/1 and 10/1) a single MPT resulted from each search, all of them showing the same topology (CI = 0.584, RI = 0.726). These cladograms are almost identical to those obtained under equal weights, except for some infrageneric relationships inside "Clade A". In all the trees obtained from the molecular matrix, *M. sabaraense* (traditionally ascribed to section *Microcochle*) is placed in "Clade A", where most species of section *Macroptilium* occur. When the monophyly of "Clade B" including *M. sabaraense* was constrained, the cladograms found after an equal weights search, differed only in seven steps (505 steps versus 498 steps) with those

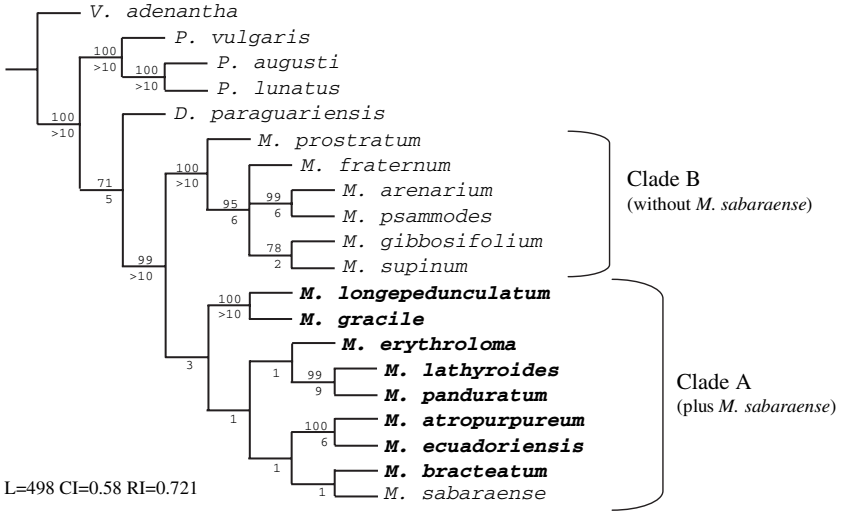

Fig. 3. Strict consensus tree of the two MPT obtained under equal weights of plastid and nuclear sequence data. Jackknife values over 50% and Bremer support values are shown above and below branches, respectively. Length (L), Consistency Index (CI) and Retention Index (RI) of the MPT are indicated. Lackey's sectional delimitation is shown in italics (sect. *Microcochle*) and bold (sect. *Macroptilium*).

obtained when no constrains were applied, or 10 steps when differential costs of 2/1 was applied (721 versus 711). However, the internal relationships of the clade did not differ in any of the searches.

Finally, under implied weighting, the same topology was found when applying different concavity constants (K = 1–6). In this cladogram, only "Clade B" is present, but without *M. sabaraense*, which conform a paraphyletic group with the remaining species of the genus (data not shown).

Combined analysis

The ILD analysis showed that the data sets were congruent ($P = 0.005$), thus a combined analysis was performed.

The three data sets previously analyzed were combined into a single matrix of 23 taxa and 1090 characters (278 parsimony informative). Under equal weights eight MPTs, 695 steps long, were obtained (CI = 0.548 and RI = 0.708). The strict consensus of these trees (Fig. 4) strongly supports the monophyly of genus *Macroptilium* (100% JK and 6 BS). The following 18 characters emerge as synapomorphies of the genus: five morphological features (calyx with upper teeth free, apical portion of the style width equal to the bottom portion, presence of an apical rotation below the stigma, pod cross-section circular, and wing petals larger than the standard petal), three unique electrophoretic seed protein bands (number 5, 27 and 36, see Appendix 3) and 10 nucleotide sites of the ITS regions.

Again, two main groups are recognized. "Clade A" contains nine species, with *M. panduratum* placed in it. Nine synapomorphies grouped this clade; three morphological characters (the tubular calyx, a nectariferous disc with lateral projection and the margo not prominent) and six nucleotide sites from the ITS sequences. "Clade B" includes the other nine species of the genus (*M. sabaraense* appears in this group), though with low support values (57% JK and 3 BS). Ten synapomorphies are found. Four of them are morphological features: stigma not globose, 6 or fewer ovules, flower size below 12 mm and inflorescence peduncle without bracts; one is an electrophoretic seed protein band; and five are nucleotide bases of ITS.

Under implied weighting, the same topology was found when applying different concavity constants (K = 1–6), and this topology agrees with the cladogram of Fig. 4, but in this case, "Clade B" is better resolved (tree not shown).

Discussion and conclusions

All of the analyses supported the monophyly of the genus, with excellent support values (99–100%). Aspects of floral morphology are the main contributors to the differentiation of the genus from related species of Phaseolinae, such as the upper teeth of the calyx, the shape of the style and stigma, and the size of the wing petals, which are larger than the standard this latter feature gave name to the genus (Bentham, 1865).

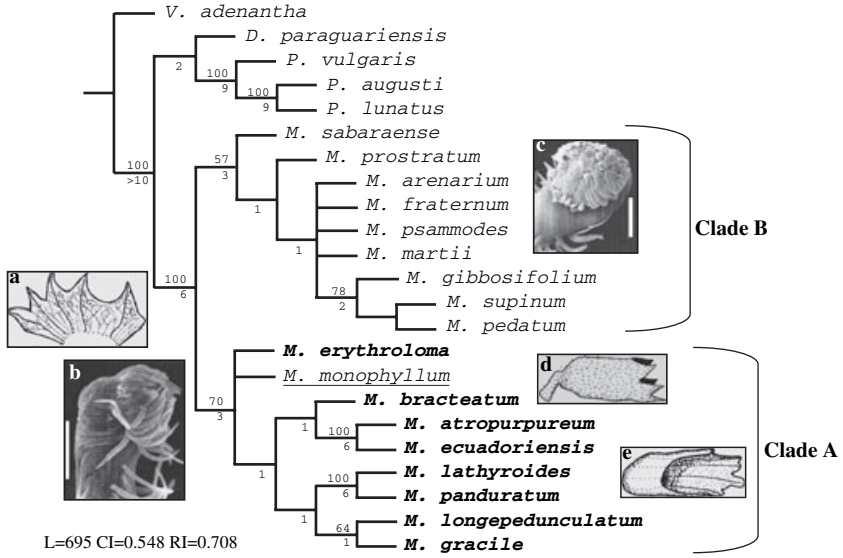

Fig. 4. Strict consensus of eight MPT found in the combined analysis (morphological, biochemical and molecular data) under equal weights. Jackknife values over 50% and Bremer support values are shown above and below branches, respectively. Length (L), Consistency Index (CI) and Retention Index (RI) of the MPT are indicated. Lackey's sectional delimitation is shown in italics (sect. *Microcochle*), bold (sect. *Macroptilium*) and underlined (sect. *Monophyllum*). The pictures show some of the synapomorphic characters of the genus and its sections: (a) calyx teeth free; (b) apical rotation below the stigma; (c) plain stigma; (d) tubular calyx; (e) nectariferous disc with big lateral projection.

In almost all the cladograms obtained here, two monophyletic groups are recovered within *Macroptilium*, with several synapomorphies and good support values. Each of them is composed by species assigned by Lackey (1983) to section *Macroptilium* (''Clade A'') and to section *Microcochle* (''Clade B''). The species of Lackey's third section (*M. monophyllum*) are placed within ''Clade A''. Each section was previously distinguished by habit, flower size and shape of the vexillary stamen (Drewes, 1997). However, results obtained here suggest that sections *Macroptilium* and *Microcochle* should instead be diagnosed by attributes from the inflorescence, calyx, stigma and pollen grains. A tubular calyx and the lateral projection of the nectariferous disc are synapomorphies of section *Macroptilium*, while a plain stigma diagnosed those species belonging to section *Microcochle*.

Two taxa, *M. panduratum* and *M. sabaraense*, showed alternative placements in the cladograms depending on the data set and the search strategy applied. The placement of the first species, assigned to section *Macroptilium*, is not resolved in the consensus tree of the morphological analysis under equal weights (Fig. 1); nevertheless, in 13 of the 26 MPTs this taxon appears in ''Clade A''. Under implied weighting of the same data set, *M. panduratum* is the basal species in the section *Macroptilium* clade (''Clade A''). However, in the combined analyses, *M. panduratum* is placed unequivocally inside ''Clade A'' (Fig. 4). The position of this taxon is surprising, as it appears as sister to *M. lathyroides*. These two species are not at all morphologically similar, and their geographic distributions are quite different: *M. panduratum* grows in Brazil, Paraguay and Argentina, while *M. lathyroides* is distributed throughout tropical America. This subclade is supported only by nucleotidic sites from the ITS sequences. Further analyses adding new data are required in order to clarify the position of *M. panduratum* inside section *Macroptilium*.

The morphological data set supports the inclusion of *M. sabaraense* in ''Clade B'' (section *Microcochle*), but the biochemical and molecular characters do not agree with these results as the taxon appears in ''Clade A'', along with species of section *Macroptilium*. However, when a new search that constrained the monophyly of section *Microcochle* including *M. sabaraense* was performed on these data sets, the MPT length did not differ substantially from those obtained without the constrains. Moreover, the combined analysis always resulted in a monophyletic ''Clade B'', including *M. sabaraense* as the most basal taxon (see Fig. 4). The species shares a number of morphological characters with species of section *Microcochle*, but the wide vexillary stamen and the seed shape are similar to those of section *Macroptilium*, which could explain its alternative placement in the cladistic analyses. It must be pointed out that one of the synonyms for *M. sabarense* is *Phaseolus prostratus* Bentham var. *longepedunculatus* Micheli, a name that is also synonymous with *M. longepedunculatum* (Mart. ex Benth.) Urb., species assigned to section *Macroptilium*. The addition of more morphological features, especially from palynological data, would help to elucidate the position of *M. sabaraense* within genus *Macroptilium*.

According to the results of the present analysis, *M. monophyllum*, assigned to a new monotypic section by Lackey (1983), is related to *M. erythroloma* (section *Macroptilium*) but with poor support values (< 50% JK). Both species share a subterminal stigma. It is important to note that Hassler (1923) described a variety of *Phaseolus monophyllus* Benth. (basionym of *M. monophyllum*), which is now assigned to *M. erythroloma*. Based on the present results, the inclusion of *M. monophyllum* in section *Macroptilium* would be the most appropriate treatment.

The relationships observed between some *Macroptilium* species are worth emphasizing. *Macroptilium longepedunculatum* and *M. gracile* always appear as a monophyletic group, and the protein electrophoretic patterns are indistinguishable (data not shown). There is no agreement on how to treat these two species, as some authors state that they are dissimilar enough as to considered them two different taxa (Maréchal et al., 1978; Pengelly and Eagles, 1995), while others think that they are extreme forms of a single species only differentiated by leaflet shape (Piper, 1926; Barbosa Fevereiro, 1986). The results obtained here are in agreement with the latter view.

M. ecuadoriensis was recently raised from a variety of *M. atropurpureum* to the level of species (Delgado Salinas and Torres Colin, 2004). The authors based their decision on the floral morphology of the two taxa. The high molecular affinity between *M. atropurpureum* and *M. ecuadoriensis*, along with the observation that they always are grouped in a monophyletic clade with high support values (100% JK and 6 BS) raises doubts about whether to consider the taxa as a single species or two.

Lastly, we have to mention the presence of a three-species clade in all the analyses, formed of three North American species: *M. gibbosifolium* (Ortega) A. Delgado, *M. supinum* (Wiggins & Rollins) A. Delgado & L. Torres-Colin, and *M. pedatum*. The other species belonging to Sect. *Microcochle* that appear within ''Clade B'' are distributed exclusively in South America.

This study is the first one to analyze all the species of the genus from a cladistic point of view, involving different sources of characters and different searching strategies. Present results allow us to confirm the monophyly of genus *Macroptilium*, with excellent support values. In addition, the subdivision in two sections: *Macroptilium*, with species *M. atropurpureum*, *M. bracteatum* (Nees & C. Mart) Maréchal & Baudet,

M. erythroloma, *M. ecuadoriensis*, *M. gracile*, *M. lathyroides*, *M. longepedunculatum*, *M. monophyllum* and *M. panduratum*; and section *Microcochle*, formed by *M. arenarium* (Bacigalupo) S.I. Drewes & R.A. Palacios, *M. fraternum, M. gibbosifolium*, *M. martii*, *M. pedatum*, *M. prostratum, M. psammodes* (Lindm.) S.I. Drewes & R.A. Palacios, *M. sabaraense* and *M. supinum*, would be the most appropriate treatment of the genus.

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Appendix 1

Accessions of Macroptilium and outgroup taxa analyzed for morphological ([m]), biochemical ([b]) and sequence data ([dn] nuclear DNA and [dc] chloroplast DNA).

Taxon	Origin of material	Accession no. and herbarium acronyms	GenBank accession
Dolichopsis paraguariensis Hassl.	Paraguay: Pres. Hayes	Palacios 1513 (BAFC)[m b]	–
	Argentina: Corrientes	Ahumada 1741 (MEXU)[dn]	AY508744*
Macroptilium arenarium (Bacigalupo) S.I. Drewes & R.A. Palacios	Argentina: Entre Ríos	Hoc 373 & 374, Palacios 1298 & 1299 (BAFC) [m b dn dc]	DQ888777/78/88
M. atropurpureum (Sessé & Moc. ex DC.) Urb.	Colombia: Tolima	CIAT 596 (CIAT) [m b dc]	DQ888799
	Mexico: Jalisco	Delgado Salinas et al. (1999)[dn]	AF115138†
M. bracteatum (Nees & C. Mart.) Maréchal & Baudet	Argentina: Salta	Palacios 769 (BAFC) [m b dc]	DQ888789
–	Argentina: Entre Ríos	Palacios 1275, Drewes 501 to 503 (BAFC) [m b dn]	DQ888767/79
M. ecuadoriense (Sessé & Moc. ex DC.) L. Torres-Colin & A. Delgado	Ecuador: Pichincha	Blasco 1778 (MEXU) [m dn]	AY508736*
M. erythroloma (Mart. ex Benth.) Urb.	Colombia: Cauca	Delgado s.n. (MEXU) [dn]	AF069117†
	Argentina: Chaco	Palacios 892 (BAFC) [m b]	–
	Argentina: Misiones	Palacios 878, 1100, 1284&1286 (BAFC)[m b]	–
M. fraternum (Piper) Juárez & S. Pérez	Argentina: Jujuy	Palacios 1014 (BAFC) [m b dn]	DQ888768/80
	Argentina: Salta	Palacios 773, 767, 775 & 781 (BAFC); Echeverry 60-66[mb] (MCNS)	–
	Argentina: Tucumán	Palacios 1044 (BAFC) [m b dc]	DQ888790
M. gibbosifolium (Ortega) A. Delgado	México: Durango	697 (BR) [m b dn dc]	DQ888769/81/91
M. gracile (Poepp. ex Benth.) Urb.	México: Chiapas	Delgado 2501 (MEXU)[dn]	AY508739*
	Colombia: Vichada	CIAT 4017 (CIAT) [m b]	–
M. lathyroides (L.) Urb.	Paraguay: Pres. Hayes	Palacios 1518 (BAFC) [m b dc]	DQ888792
	Argentina: Chaco	Palacios 891 (BAFC) [m b dn]	DQ888770/82
M. longepedunculatum (Mart. ex Benth.) Urb.	Panamá: Panamá	CIAT 4169 (CIAT) [m b]	–
	Argentina: Corrientes	Palacios 910 (BAFC) [m b dn]	DQ888771/83
	Argentina: Entre Ríos	Palacios 902 & 908 (BAFC) [m b dc]	DQ888793
M. martii (Benth.) Maréchal & Baudet	Paraguay	Rojas T. 1102 (FCQ)[m]	–
M. monophyllum (Benth.) Maréchal & Baudet	Paraguay	Balansa B. 1501 (G)[m]	–
M. panduratum (Benth.) Maréchal & Baudet	Argentina: Salta	Palacios 1357, Hoc 341 (BAFC)[m b dn dc]	DQ888772/84/94
M. pedatum (Rose) Maréchal & Baudet	México	Pringle C. 8367 (US) [m]	–
M. prostratum (Benth.) Urb.	Argentina: Misiones	Palacios 1287 (BAFC) [m b dn]	DQ888773/85
	Argentina: Corrientes	Palacios 1073 (BAFC) [m b dc]	DQ888795
	Argentina: Entre Ríos	Palacios 844, 856, 1205 & 1274 (BAFC) [m b]	–
M. psammodes (Lindm.) S.I. Drewes & R.A. Palacios	Argentina: Misiones	Palacios 1282 (BAFC) [m b dn]	DQ888774/86
	Argentina: Corrientes	Palacios 1090, 1279 & 1280 (BAFC) [m b dc]	DQ888796
M. sabaraense (Hoehne) V.P. Barbosa ex G.P. Lewis	Brasil: Minas Gerais	852 (BR)[m b dn dc]	DQ888776/97
M. supinum (Wiggins & Rollins) A. Delgado & L. Torres-Colín	USA: Arizona	872 (BR)[m b dn dc]	DQ888775/87/98
Phaseolus agusti Harms	Perú	Nunez 7081 (MEXU)[dn]	AF115180†
	Argentina: Salta	Hoc 281 & 282 (BAFC)[m b]	–

Appendix 1. *Continued*

Taxon	Origin of material	Accession no. and herbarium acronyms	GenBank accession
P. lunatus L.	Colombia: Magdalena	CIAT 26509 (CIAT)[dn]	AF115175†
	Argentina: Jujuy	Palacios 1210 (BAFC)[m b]	–
P. vulgaris L.	Argentina	CIAT 19889 (CIAT)[dn]	AF115166†
	Argentina: Jujuy	Palacios 1010 (BAFC)[m b dc]	DQ888800
Vigna adenantha (G. Mey.) Maréchal, Mascherpa & Stainier	Colombia	CIAT 4022 (CIAT)[dn]	AF069119†
	Argentina: Jujuy	Palacios 1209 (BAFC)[m b]	–
	Argentina: Chaco	Palacios 896 (BAFC)[m b]	–

Authorship of sequences gathered from GenBank: *Riley Hunting et al. (2004) and †Delgado-Salinas et al. (1999).

Appendix 2

Morphological matrix.

	0123456789	1 0123456789	2 0123456789	3 0123456789	4 0123
V. adenantha	00?*00?110	100010000?	?000000110	?000??0020	1010
P. vulgaris	0100000110	00010000??	0000000000	000000*$*1	0100
P. augusti	0100000110	0000000000	0000001000	00000001*1	0100
P. lunatus	0100000110	0000000000	0000001000	00000001*1	0100
D. paraguariensis	0010000010	1000000000	0000000000	10111000*0	1000
M. arenarium	1000111101	1011000010	0111111001	020?012120	1011
M. bracteatum	0000000121	0101011111	2000110101	1100101010	1010
M. fraternum	1001101101	1001100010	0111111001	0210012220	1011
M. gibbosifolium	1001100100	1011000010	0111111001	0211010120	1011
M. lathyroides	0010000111	0101111111	2000110111	1000101000	1010
M. longepedunculatum	0010011110	0101111111	2000110111	1000101000	1010
M. panduratum	0000111100	0101100011	1000110011	0001000100	1010
M. prostratum	1000000000	0001000010	0110111001	0211010120	1011
M. psammodes	1000000101	1011000010	0111111001	0202010120	1011
M. sabaraense	0001000100	0001000011	011?111001	00?????????	???1
M. supinum	1001111100	0011000?10	????111001	02????????0	1011
M. ecuadoriensis	0001100111	0111011111	??0??10111	10??101010	1010
M. atropurpureum	0001100111	0111011111	??0?110111	1001101010	1010
M. erythroloma	0000000120	0101000011	1021110001	0000101010	1010
M. gracile	0010000110	0101111111	2000110111	1000101000	1010
M. monophyllum	0010000010	0101000011	1?2?110101	00??1?????	???0
M. martii	0000000101	1011000010	0???111001	00?????????	???1
M. pedatum	1001111100	0011000010	0???111001	02??0?????	???1

* = Polymorphism, states 0 & 1; $ = polymorphism, states 1 & 2; ? = missing data.

Morphological characters and their states

0. Habit: erect or climber plants (0); prostrate plants (1)
1. Plant indumentum: hairs not hook-shaped (0); hairs hook-shaped (1)
2. Stem structure: solid, stuffed (0); fistular, tubular (1)
3. Tuberosus root system: absent (0); present (1)
4. Adventitious roots on stem nodes: absent (0); present (1)
5. Hypogeal racemes (inflorescences growing or remaining below ground): absent (0); present (1)
6. Cleistogamous flowers (i.e., fertilized within the unopened flower): absent (0); present (1)
7. Leaflet consistency: coriaceus, leathery (0); membranous-papyraceus (1)
8. Bracts on the peduncle of the inflorescence: absent (0); present, basally on the peduncle (1); present, above the base of the peduncle (2)
9. Inflorescence apex: not comosus (0); comosus, i.e., bearing a tuft of bract hairs (1)
10. Internodes of the bud portion of the inflorescence: long (0); short (1)
11. Calyx shape: campanulate (0); tubular, see picture *d* of Fig. 4(1)
12. Calyx teeth: shorter than the tube (0); equal or longer than the tube (1)
13. Calyx upper teeth: partially or completely fused (0); free, see picture a of Fig. 4(1)
14. Border of the standard petal: emarginate, shallowly notched (0); straight (1)

15. Standard petal shape: orbicular (0); obovate or oblong (1)
16. Standard petal claw (lower portion of the petal): well differentiated from the rest of the petal (0); slightly differentiated (1)
17. Size of the standard petal auricles (small lobes located on the base of the petal): large, distinctive (0); small, indistinguishable (1)
18. Wing petals size: equal o smaller than the standard petal (0); larger than the standard petal (1)
19. Basal portion of the vexillary stamen: geniculate, i.e., bent abruptly like a knee (0); not geniculate (1)
20. Shape of the nectariferous disc of the flowers: perfectly cylindrical (0); with a short lateral projection (1); with a big lateral projection, see picture *e* of Fig. 4(2)
21. Stigma surface: globosus, spherical (0); plain, see picture *c* of Fig. 4(1)
22. Stigma position: apical (0); lateral (1); subapical (2)
23. Hair ring around the stigma: complete (0); incomplete (1)
24. Style shape: with its base or apex dilated (0); filiform (1)
25. Apical rotation of the style just below the stigma: absent (0); present, see picture *b* of Fig. 4(1)
26. Ovule number: more than 6 (0); 1–6 (1)
27. Pod shape: falcate, curved like a sickle (0); straight (1)
28. Pod arrangement on the inflorescence axis: adpressus, i.e., lying flat against the axis (0); patens, i.e., diverging from the axis inflorescence at almost 90° (1)
29. Pod cross-section: laterally flattened (0); circular (1)
30. Pod dehiscence: non-shattering (0); shattering (1)
31. Seed shape: oblong to elliptic (0); obovate (1); orbicular (2)
32. Germination mode: epigeal, growing upon the ground (0); hypogeal, growing or remaining below ground (1)
33. Primordial leaves: with entire stipules (0); with bifid stipules (1); with double stipules (2). (Terminology according Baudet, 1974)
34. Pollen grain: suboblate (0); spherical (1)
35. Amb (polar view of the pollen grain): circular (0); subtriangular to triangular (1)
36. Margo (reinforced border of aperture): prominent (0); not prominent (1); absent (2)
37. Pollen aperture type: tricolporate with large colpi (0); tricolporate with small colpi (1); porate (2)
38. Pollen exine sculpture: reticulate (0); microreticulate (1); other (2)
39. Inflorescence nodes: nectariferous (0); non-nectariferous (1)
40. Flower bracts: persistent (0); caducous (1)
41. Pedicels/calyx length (at anthesis): pedicel shorter than the calyx tube (0); pedicel larger than the calyx tube (1)
42. Bracteoles: persistent (0); caducous (1)
43. Flower size, as measured by the length of the wing: larger than 12 mm (0); smaller than 12 mm (1)

Appendix 3

Biochemical matrix.

	0123456789	1 0123456789	2 0123456789	3 0123456789
V. adenantha	1100000000	0000000000	0001000001	0000000000
P. vulgaris	0000001110	0001000000	0101000010	1011000000
P. augusti	0000001110	1001000100	0101000010	1111000000
P. lunatus	0000000100	1001000100	0101000010	0111000000
D. paraguariensis	0000000000	0000000000	000000000?	0000000000
M. arenarium	1101010000	0000000000	000?0???0?	0000001000
M. bracteatum	1100110001	0010010101	1011000101	0000001101
M. fraternum	1100010000	0100000000	0001011101	0000001000
M. gibbosifolium	??01010000	0000001010	000101110?	0000101000
M. lathyroides	1110010000	0000010000	1001100101	0000001101
M. longepedunculatum	1100110001	0000000000	000100010?	0000001010
M. panduratum	110011000?	0100110010	001000010?	0000001000
M. prostratum	1101010000	0100001010	0001011101	0000011000
M. psammodes	1101010000	0100001010	0001011101	0000011000
M. sabaraense	??0??10001	0100010000	000100010?	0000001000
M. supinum	??01010000	0100001010	0001011101	0000101000
M. atropurpureum	1100010000	0000100100	101100010?	0000001101
M. erythroloma	1110010000	0010010101	1001100101	0000001000
M. gracile	1100110001	0000000000	000?000?0?	0000001010

?: Missing data.

The matrix represents 40 of 141 biochemical characters, where presence (1) and absence (0) of seed protein bands in electrophoretic gels were scored for 19 species. Only parsimony informative characters are included. Those species whose biochemical profiles could not be obtain, were treated as missing data.