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Introduction to the phylogenetic analysis of *Maxillaria* Ruiz & Pav. (Maxillariinae, Orchidaceae)

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Abstract: *Maxillaria* Ruiz & Pav. is the largest genus of the subtribe Maxillariinae Lindl. (Orchidaceae) and also one of largest genera within the subfamily Vandoideae Endl. *Maxillaria* contains mainly tropical and subtropical orchids. It is a highly disorderly genus because of the great number of species as well as a multitude of features occurring in many combinations. Both the number of species assigned to this genus, and the infrageneric classifications of *Maxillaria*, are not clearly resolved yet. In this paper, a phylogenetic study based on Internal Transcribed Spacer (ITS) sequences is presented. The results show the monophyletic character of the subtribe Maxillariinae and the paraphyletic character of *Maxillaria*.

Key words: Orchidaceae, Maxillariinae, *Maxillaria*, ITS, taxonomy, phylogenetic relationship

1. Introduction

The subtribe Maxillariinae comprises taxa widely distributed within the area of tropical America, with most of them clustered in the genus *Maxillaria* Ruiz & Pav. *sensu* Dressler. Maxillarias range from south Florida and Mexico, throughout Central America, to Argentina, with the highest diversity in the Andean region. They can be found at any elevation up to the snow line, growing in a wide range of ecosystems: from seasonally dry forests to moist wet forest or cloud forest. The majority of species are epiphytes, but they also may grow as lithophytes or even terrestrial plants in open environments. The genus is species-rich but the precise number of taxa assigned to *Maxillaria* is still unknown and notably depends on adopted classification. Dressler (1993) estimated the number of true species of this genus as 420, while Index Kewensis lists 634 names within the genus, of which 80 are probably synonymic, and 564 could be accepted as true names. Results of later research in Costa Rica (Atwood & Mora de Retana 1999) raised the number to more than 600 true names. Finally, Senghas (2002) specified 750 *Maxillaria* species.

This genus is characterized by a combination of the following features: conduplicate leaves, usually 1-flowered inflorescence, presence of column foot, 4 pollinia, and viscidium generally horseshoe-shaped. The significant unification of flower structures can be observed as well as a very high variability of the vegetative characters, such as: plant size and type, model of growth, leaf number and type, and inflorescence type. It must be noted that such a great morphological variability can be due to adaptation to local habitats, and the unification of the flower structures may result from convergence as an effect of adaptation to a similar group of pollinators.

The lack of clearly defined delimitations within the subtribe Maxillariinae causes many controversies with generic and infrageneric classifications of all genera assigned to this subtribe. Existing classifications, based mainly on morphological features (Christenson 2002; Senghas 1993, 2002; Dressler 1993), are fragmentary and contradictory at many points.

Recently, studies based on nucleic acid data have been often used to infer relationships among groups of plants, including many groups of orchids. This study is based on ITS (Internal Transcribed Spacer) sequences, which have been successfully used in many phylogenetic analyses of closely related taxa, from the subtribe level (Douzery *et al*. 1999; Cameron & Chase 1999) to the generic level (Whitten *et al*. 2000; Gravendeel *et al*. 2001). The ITS region is a part of nuclear ribosomal DNA (nrDNA), a multigene family occurring in the genome in many copies. The copies, repeated tandemly

at chromosomal loci, consist of highly conservative regions encoding ribosomal subunits (5,8S, SSU, LSU) and much more variable non-coding parts (ITS and IGS, which stands for Intergenic Spacer) (Baldwin 1992).

The main goal of our study is to clarify relationships between species of the subtribe Maxillariinae, as that could help to ascertain the rightness of separating the genera within this subtribe and to form an infrageneric classification of the genus *Maxillaria*. In this paper we present evidence gathered by analysis of the ITS region for 34 orchid taxa ever assigned to *Maxillaria*. This was a preliminary work, and subsequently we intend to analyze ITS data for other species of *Maxillaria*. In the future, a combination of molecular and morphological data will form a basis for creating an infrageneric classification of *Maxillaria sensu lato*.

Table 1. Sources of plant material for the taxa included into the study

Taxon	Voucher data	Accession number
Maxillaria chrysantha Barb. Rodr.	Ecuador, DLS 451	DQ924384
Maxillaria coccinea (Jacq) L.O.Wms.	DLS 380	DQ924385
Maxillaria consanguinea Klotzsch	DLS 418	DQ924386
Maxillaria cucullata Lindl.	Ecuador, DLS 405	DQ924387
Maxillaria densa Lindl.	Ecuador, DLS 406	DQ924388
Maxillaria elatior Rchb.f.	DLS 379	DQ924389
Maxillaria houtteana Rchb.f.	Ecuador, DLS 399	DQ924391
Maxillaria madida Lindl.	DLS 377	DQ924392
Maxillaria marginata Fenzl.	Ecuador, DLS 393	DQ924393
Maxillaria mathewsii Lindl.	Ecuador, DLS 400	DQ924394
Maxillaria pachyphylla Schltr. ex. Hoehne	Ecuador, DLS 394	DQ924395
Maxillaria paulistana Hoehne	Heidelberg 122352	DQ924396
Maxillaria ponerantha Rchb.f.	Ecuador, DLS 412	DQ924397
Maxillaria praestans Rchb.f.	Ecuador, DLS 411	DQ924398
Maxillaria procurrens Lindl.	DLS 388	DQ924399
Maxillaria ramosa Ruiz & Pav.	DLS 383	DQ924401
Maxillaria ruberrima Garay	DLS 374	DQ924402
Maxillaria rufescens Lindl.	Ecuador, DLS 395	DQ924403
Maxillaria santanae Carnevali & I.Ramirez	DLS 387	DQ924404
Maxillaria schunkeana Campacci & Kautsky	DLS 375	DQ924405
Maxillaria similis Garay & Dunst.	DLS 382	DQ924412
Maxillaria sp.	DLS 371	DQ924390
Maxillaria sp.	Ecuador, DLS 408	DQ924400
Maxillaria tenuifolia Lindl.	DLS 384	DQ924413
Maxillaria ubatubana Hoehne	Ecuador, DLS 415	DQ924411
Maxillaria uncata Lindl.	DLS 389	DQ924406
Maxillaria valenzuelana (A.Rich.) Nash	DLS 372	DQ924407
Maxillaria variabilis Batem. ex. Lindl.	DLS 390	DQ924408
Maxillaria vitelliniflora Barb.Rodr.	Heidelberg 121052	DQ924410
Maxillaria vernicosa Barb.Rodr.	DLS 385	DQ924409

2. Material and methods

2.1. Plant samples

Fresh leaf samples of 30 taxa (Table 1) were collected at flowering time. Species selected for this analysis were classified to the genus *Maxillaria s.l.* (currently genera *Cryptocentrum* and *Trigonidium* are excluded from the genus *Maxillaria*). The materials were obtained from species cultivated at the Heidelberg Botanical Garden, from private collections of Prof. D. L. Szlachetko, or were collected in Ecuador by Prof. D. L. Szlachetko. Sequence information of additional 4 ingroup taxa and 3 outgroup taxa were taken from NCBI resources (accession numbers in Table 2).

2.2. DNA isolation, PCR amplification, and sequencing

Total DNA was extracted from 100 mg of fresh or 20 mg of silica-dried leaf material with the Genomic Mini AX Plant kit (A&A Biotech, Poland), following manufacturer's instructions, and then stored at -20^{o}C for later use. The ITS1, 5.8S, and ITS2 regions were amplified by polymerase chain reaction (PCR) with reagent volume of 50 µL, using 1.25 units of the Blue Perpetual™ Taq DNA Polymerase (Eurx, Poland), and 100 ng of the 2 primers designed by G. Sheridan (University of Bath) and published by Douzery *et al.* (1999): a forward primer (AB101) annealing in the 18S gene, 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG, and a reverse primer (AB102) annealing in the 26S gene, 5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC. The PCR protocol comprised 30 cycles, starting with 5 min

of initial premelt at 94°C, then each cycle with 45 s of denaturation at 94°C, 45 s of annealing at 52°C, and 1 min of extension at 72°C, with a final extension for 7 min at 72°C. PCR products were then cleaned with the High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Germany), following the manufacturer's protocol. Purified PCR products were sequenced by using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, UK). Cycle sequencing conditions were as follows: 20 sec of initial denaturation followed by 25 cycles, each with 15 sec of denaturation at 94°C, 20 sec of annealing at 52°C, and 1 min of elongation at 60°C. Sequencing reactions were purified by ExTerminator (A&A Biotech, Poland), following the manufacturer's protocol. Pelleted samples were sequenced on an Applied Biosystems 377 automated sequencer, with both strands (upstream and downstream) to assure accuracy in base calling. Sequences were edited in FinchTV (Geospiza, Inc), and sequencing artifacts at the 5' and 3' ends were removed.

(24%) characters were parsimony-informative. The heuristic parsimony search yielded 1111 equally parsimonious trees with a length of 683 steps, consistency index (CI) of 0.706, and retention index (RI) of 0.748. Fig. 1 depicts a consensus tree of all most parsimonious tress found; numbers above branches represent bootstrap values above 50%. There are 5 clades within the ingroup, with bootstrap support higher than 50%: 2 with high support (more than 90%), and 3 with moderate support (66-85%).

As shown in Fig. 1, members of the ingroup form a monophyletic clade. *Maxillaria s.l.* is paraphyletic – the known accepted genera (*Trigonidium*, *Cryptocentrum*) as well as the genera formerly separated from *Maxillaria* (*Camaridium*, *Ornithidium*, *Sepalossacus*, *Mersupiaria*, *Heteroaxis*) are anchored within the genus.

The species *M. houtteana, M. tenuifolia*, *M. variabilis, M. elatior* form a highly supported clade (BP 97), with an also highly supported sister group (BP 88):

Table 2. Sequences obtained from the GenBank

Taxon	Accession number
Maxillaria umbratilis L. O. Wms.	AF239331
Maxillaria violacepunctata Rchb. f.	AF239332
Cryptocentrum calcaratum (Schltr.) Schltr.	AF239413
Cyrtopodium andersonii (Lamb. ex A. L. Andrews) R. Br.	AF470490
Eulophia guineensis Lindl.	AF239413
Eulophia graminea Lindl.	AF284727
Trigonidium egertonianum Bateman ex Lindl.	DQ210211

2.3. Sequence alignment

A raw alignment was accomplished by using ClustalX 1.8 with default settings, and then adjusted manually. The final data set was analyzed with PAUP version 4.0b4a (Swofford 2000) with *Cyrtopodium andersonii* (Lamb. *ex* A. L. Andrews) R. Br., *Eulophia graminea* Lindl., and *Eulophia guineensis* Lindl., designated as outgroup taxa. Full heuristic search was performed with optimality criterion set to parsimony, tree-bisection-reconnection (TBR) branch swapping, MULTREES option in effect, simple addition, and ACCTRAN optimization. All characters were unordered and equally weighted. For the maximum parsimony analysis, gaps in aligned sequences were treated as missing data, and all characters were treated as unordered (nonadditive). Internal support of clades was evaluated by the bootstrap (Felsenstein 2004) with 1000 bootstrap replicates.

3. Results and discussion

The length of the aligned matrix was 786 base pairs, of which 427 sites (54%) were constant, 169 (22%) variable sites were parsimony-uninformative, and 190

M. ponerantha, M. procurrens and *M. similis*. Both Christenson (2002) and Senghas (2002) assigned these species to different genera. In our opinion further investigations with identification of possible morphological synapomorphies are required to resolve this problem.

The specimens classified by Christenson (2002) into *Maxillaria* sect. *Urceolatae*, form a moderately supported clade (BP 66). Recently, this section has been raised to the generic status *Christensonella* (Szlachetko *et al.* 2006), with our results confirming this separation and also the monophyletic character of the new genus.

The next highly supported clade (BP 94) consists of *M. coccinea, M. ruberrima* (grouped together in the genus *Ornithidium* Salisb.) and *M. ramosa* (genus *Sepalosaccus* Schltr.). Together they form also a highly supported clade (BP 99) with *M. valenzuelana* (genus *Mersupiaria* Hoehne) and *M. violacepunctata* (genus *Heterotaxis* Lindl.) and *M. santanae* (*Maxillaria* sect. *Iridioliae*). To resolve this problem, the boundaries and ranks of the taxa mentioned above should be revised.
The taxa assigned to *Maxillaria* sect. *Repantes* Pfitz. (*M. consangiunea*, *M. ubatubana*, *M. chrysantha*, *M. marginata*, *M. schunkeana*) form one highly supported

clade (BP 100) with the moderately supported *Cryptocentrum* (this genus in some classifications is included in *Maxillaria*) as a sister group (BP 82). This section seems to be monophyletic but further studies are necessary to specify the relationships with genus *Cryptocentrum*.

Christenson (2002) placed *M. cucullata* and *M. praestans* in *Maxillaria* section *Cucullatae*, while *M. densa* in *Ornithidium* Salisb. and *M. umbratilis* in *Camaridium* Lind. Our results do not support this classification since those 4 taxa form single highly supported clades (BP 100). *M. praestans* seems to be a synonym

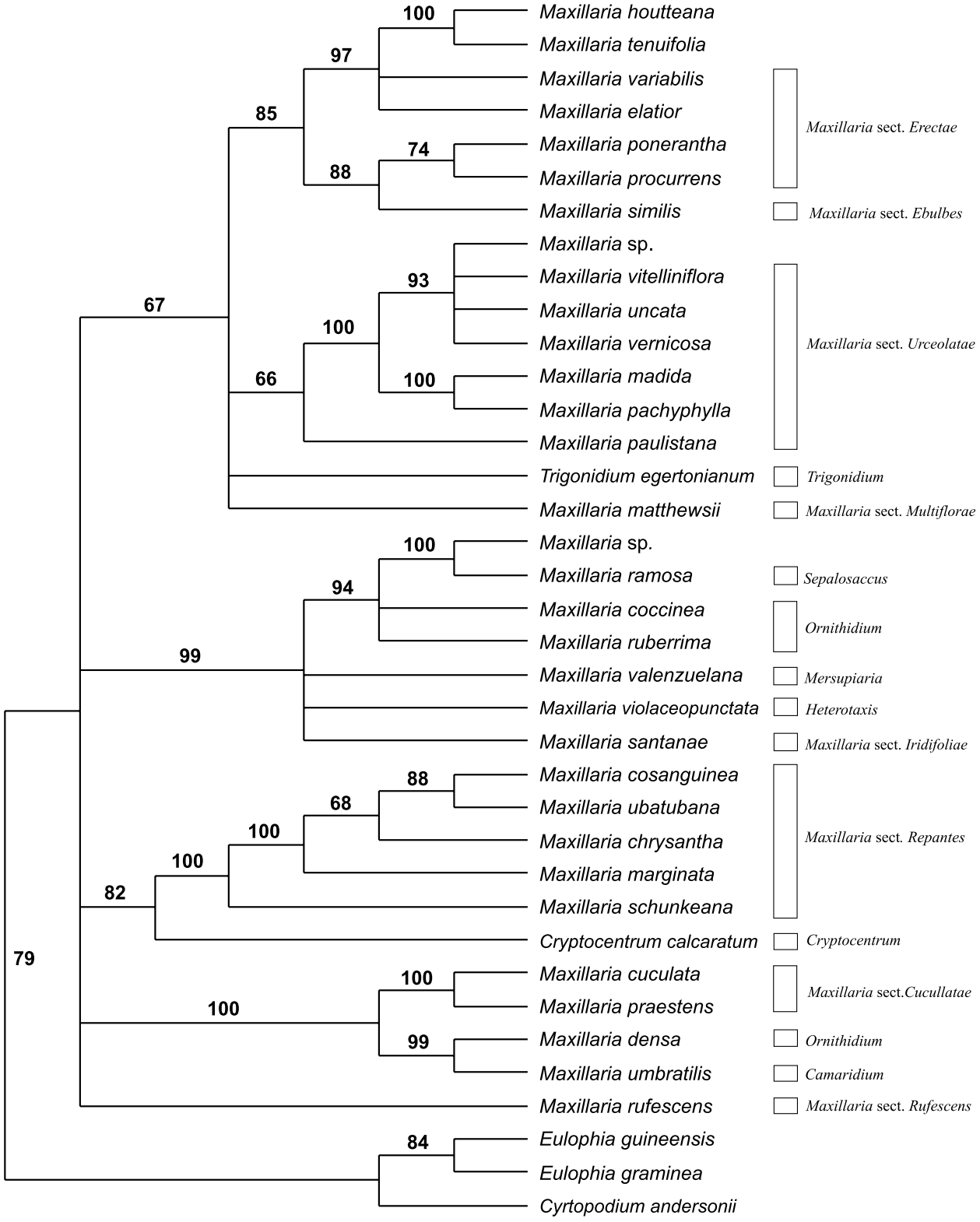

Fig. 1. Consensus tree of 1111 most parsimonious trees. Bootstrap percentages >50% are listed above branches. Taxonomic classification after Christenson (2002) and Senghas (1993)

of *M. cucullata*, as both species have the same ITS region.

There are 3 unresolved clades: *M. rufescens* (included by Senghas together with *M. cucullata* in Artengruppe XI), *Trigonidium egertonianium* and *M. mathewsii*. It is noteworthy that *Trigonidium*, by some authors assigned to *Maxillaria*, is placed among species of *Maxillaria*.

The taxa included into *Ornithidium* Salisb. – *M. coccinea* and *M. ruberrima, M. densa* – are separated into 2 clades, so this genus seems to be paraphyletic.

4. General conclusions

Our study confirms the conclusions of previous studies, that the ITS region seems to be a very useful source for studies of phylogenetic relationships within subtribes (Douzery *et al*. 1999; Cameron & Chase 1999) and genera (Whitten *et al*. 2000; Gravendeel *et al*. 2001). Maxillariinae form a monophyletic group (Whitten *et al*. 2000) with high support, but relationships between the genera assigned to this group are still complicated. The great morphological divergence and the large number of species are the reasons why contradictory classification systems have been created (Dressler 1993; Christenson 2002; Senghas 1993, 2002). With the aid of information obtained from DNA studies, the future revision of genus *Maxillaria sensu lato* should help to clarify those relationships and enable the creation of one final classification.

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References

Atwood H. H. & Mora de Retana D. E. 1999. Orchidaceae: Tribe Maxillariineae: Subtribes Maxillariinae and Onciidinae. Fieldiana 40: 1-182.

Baldwin B. G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: en example from the Compositae. Molecular Phylogenetics Evolution 1: 3-16.

Cameron K. M. & Chase M. W. 1999. Phylogenetic relationship of Pogoniinae (Vanilloideae, Orchidaceae): An herbaceous example of the Eastern North America – Eastern Asia phytogeographic disjunction. Journal of Plant Research 112: 317-329.

Christenson E. A. 2002. Vue d'ensemble du genere *Maxillaria* (Orchidaceae). Richardiana 2(2): 41-65.

Douzery E. J. P., Pridgeon A. M., Kores P., Linder P., Kurzweil H. & Chase M. W. 1999. Molecular phylogenetics of Diseae (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. Am. J. Bot. 86: 887-899.

Dressler R. L. 1993. Phylogeny and classification of the orchid family. 314 pp. Dioscorides Press, Portland.

Felsenstein J. 2004. Inferring Phylogenies. 664 pp. Sinauer Associates. Sunderland, Massachusetts.

Gravendeel B., Chase M. W., Vogel E. F., Roos M. C., Mes H. M. & Bachmann K. 2001. Molecular phylogeny of *Coelogyne* (Epidendroideae; Orchidaceae) based on plastid RFLPS, *mat*K and nuclear ribosomal ITS sequences: evidence for polyphyly. Am. J. Bot. 88(10): 1915-1927.

Senghas K. 1993. Subtribus Maxillariinae. In: F. G. Brieger, R. Maatsch & K. Senghas (eds.). Rudolf Schlechter: Die Orchideen. Blackwell Wissenschafts-Verlag, Berlin.

Senghas K. 2002. *Maxillaria* (Orchidaceae), un genre chaotique. Richardiana 2(1): 29-38.

Swofford D. L. 2000. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b2. Sinauer Associates, Sunderland MA.

Szlachetko D. L., Mytnik-Ejsmont J., Górniak M. & Śmiszek M. 2006. Genera et Species Orchidalium. 15. Maxillariinae. Polish Botanical Journal 51(1): 57-59.

Whitten W. M., Williams N. H. & Chase M. W. 2000. Subtribal and generic relationships of Maxillarieae (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. Am. J. Bot. 87(12): 1842-1856.