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Comparative molecular and morphological studies in selected *Maxillariinae* orchids

Abstract

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The phylogenetic relationships within *Orchidaceae* subtribe *Maxillariinae* s.str. were investigated by Maximum Parsimony and Bayesian analyses of nuclear ribosomal ITS1 and ITS2 DNA sequences in 27 species. While the monophyly of *Maxillariinae* is supported, *Maxillaria* in its current, narrower circumscription is clearly paraphyletic, since all presently accepted genera examined *(Chrysocycnis, Cryptocentrum, Mormolyca, Trigonidium)* and the former segregates *Camaridium*, *Heterotaxis*, *Marsupiaria, Neourbania, Ornithidium* and *Pseudomaxillaria* are nested within it. *Camaridium, Heterotaxis* and *Ornithidium* are, moreover, polyphyletic. The resulting molecular trees show six more or less well supported clades but are not very well resolved in their basal parts. To study character evolution, the molecular data were compared with pollinarium morphology, using scanning electron microscopy in 22 taxa, and further morphological data. The comparison indicates that most features have evolved several times independently. In growth habit a trend from caespitose to rhizomatous is found. Palynologically three morphological lines are indicated: (1) from four greater pollinia in two pairs to four smaller, equal, separate pollinia; (2) from spherical to clavate pollinia; (3) from pollinia with rugulate (sometimes gemmate, granulate, fossulate, microfoveolate) to psilate surface. A more extensive taxon sampling is needed to decide if and how *Maxillaria* s.l. has to be divided in smaller monophyletic genera.

Key words: phylogeny, systematics, ITS, pollinarium, *Maxillaria, Orchidaceae.*

Introduction

The neotropical orchid subtribe *Maxillariinae* (cymbidioid phylad of the advanced *Epidendroideae* sensu Dressler 1993) includes 460-500 species (Atwood & Mora de Retana 1999) and is currently divided into eight genera (Dressler 1993): *Maxillaria* in a broad sense, including *Camaridium* Lindl., *Heterotaxis* Lindl., *Ornithidium* R. Br., *Marsupiaria* Hoehne, *Neourbania* Fawc. & Rendle, *Pseudomaxillaria* Hoehne and *Sepalosaccus* Schltr., and seven minor genera, *Anthosiphon* Schltr., *Chrysocycnis* Linden & Rchb. f., *Cryptocentrum* Benth., *Cyrtidiorchis* Rausch., *Mor-*

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molyca Fenzl, *Pityphyllum* Schltr. and *Trigonidium* Lindl. All members of this subtribe have conduplicate leaves but are otherwise fairly diverse in their vegetative features: most plants are epiphytes, some are terrestrial or lithophytic, and they may have pseudobulbs of a single internode or not (Dressler 1993, Atwood & Mora de Retana 1999). The inflorescence is lateral, usually single-flowered, but sometimes grouped in clusters (fascicles) (Senghas 1993-94, Atwood & Mora de Retana 1999). The flowers vary in size and colour. The lip is usually hinged to a column foot (extension of the base of the column), sometimes forming a saccate nectary with the column foot (Dressler 1993). The lateral sepals are connate to varying degrees, sometimes forming a spur. The pollinarium contains four pollinia, which are mostly attached to a horseshoe-shaped viscidium or to a more or less well-developed tegular stipe (Dressler 1993). The velamen radicum corresponds to the *Cymbidium* type (Porembski & Barthlott 1988) and the seed structure is of the *Maxillaria* type (Chase & Pippen 1988). The seeds of *Cryptocentrum* are deviating but clearly derived from *Maxillaria* (Senghas 1993-94).

Some of the former or current *Maxillaria* segregates differ only in a few features (e.g., *Heterotaxis* complex, *Marsupiaria, Mormolyca*). Presence or absence of the column foot, e.g., was used to distinguish *Maxillaria* (foot present) from *Mormolyca* (foot absent) (Garay & Wirth 1959). The degree of fusion of the lateral sepals was used to distinguish *Sepalosaccus* and *Pseudomaxillaria* from *Maxillaria* (Brieger 1977). Other segregates show atypical vegetative habits (e.g., *Cryptocentrum, Neourbania*) or unusual pollination syndromes such as pseudocopulation, which has been suggested to occur in *Chrysocycnis, Cyrtidiorchis, Mormolyca* and *Trigonidium* (Van der Pijl & Dodson 1966) and has recently been confirmed for *Trigonidium obtusum* (Singer 2002) and *Mormolyca ringens* (Singer & al. 2004).

The subtribe *Maxillariinae* is a rather poorly understood orchid group. No monograph is available and few articles on it were published. Senghas (1993-94), who took over Schlechter's system without changes, provided the most comprehensive work. The currently accepted system (sensu Dressler 1993) is based on morphological and anatomical characters, but the generic boundaries are not very convincing. In recent years some anatomical, chemical and morphological studies on a small number of *Maxillariinae* species were published (Davies & Winters 1998, Holtzmeier & al. 1998, Davies & al. 2000, Davies & al. 2003a-b, Davies & Turner 2004, Flach & al. 2004, Singer & Koehler 2004), which contribute to a clarification of the relationships in this group. Cladistic analyses of nuclear and chloroplast DNA regions within the tribe *Maxillarieae* (Whitten & al. 2000) demonstrated that *Maxillariinae* (sensu Dressler 1993) form a monophyletic group (four representatives included). Ojeda & al. (2003) published a molecular study (ITS) on the *Heterotaxis* complex. Multidisciplinary studies on Brazilian *Maxillariinae* (Singer & Koehler 2003) and a huge, mainly molecular study on the whole subtribe (Williams & Whitten 2001) are in progress. Very little is known about chromosome numbers, hybridisation and speciation in this orchid group until now. Although palynological data were already successfully applied in phylogenetic investigation in orchids (e.g., Williams 1970a-b, 1972, Williams & Broome 1976, Schill & Pfeiffer 1977, Ackerman & Williams 1981, Chase 1987, Burns-Balogh & Hesse 1988, Hesse & al. 1989, Stenzel 2000, Singer & Koehler 2004), no research seems to have been done on the pollinarium surface within this subtribe apart from Schill & Pfeiffer (1977) and Dietrich (unpubl. 1989).

The present work intends to test the monophyly of the formerly or currently accepted genera within *Maxillariinae* using the molecular marker ITS and to compare the results with palynological and other morphological data to study character evolution.

Material and methods

Plant material. – Twenty-seven species from five presently accepted and six formerly separated genera of *Maxillariinae* sensu Dressler (1993) were examined. Two *Lycaste* species (subtribe *Lycastinae*) were chosen as outgroup on the basis of previous morphological and molecular phylogenetic studies within *Maxillarieae* (Stern & al. 2004, Whitten & al. 2000). Pollinaria were

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available from twenty-two plants of the ingroup and the outgroup taxa. Pollinaria of *Maxillaria valenzuelana* were already investigated by Dietrich (unpubl. 1989) and the data used in this study. Fresh leaf material and pollinaria were mainly obtained from cultivated specimens. The investigated species are listed in Table 1. Sequences for three additional taxa were taken from EMBL (Table 2).

DNA isolation, amplification and sequencing. – DNA was extracted from 60 mg silica gel-dried (Chase & Hills 1991) and crushed leaf material according to Hellwig & al. (1999). ITS1 and ITS2 were amplified separately using the primers "ITS5" (5'-GGAAGTAAAAGTCGTAACAAGG-3') or a modification of "ITS5" (5'-GGAAGGAGAAGTCGTAACAAGG-3') and "P2" (5'-CTCGATGGAACACGGGATTCTGC-3') or "58SNR1" (5'-CGCATTTCGCTGCGCTC-3') or "58SNR2" (5'-TCGCTGCGCTCTTCATCG-3') for ITS1 and "ITS3" (5'-GCATCGATGAAGAACGCAGC-3') or "58SNF1" (5'-GAGCGCAGCGAAATGCG-3') or "58SNF2" (5'-TCGATGAAGAGCGCAGCG-3') and "ITS4" (5'-TCCTCCGCTTATTGATATGC-3') or a modification of "ITS4" (5'-CTTTTCCTCCGCTTATTGATATG-3') for ITS2. In most cases, the modified version of "ITS5" and "P2" were used to amplify ITS1, "58SNF1" and the modified "ITS4" to amplify ITS2. For primers "ITS3", "ITS4" and "ITS5" see White & al. (1990), for primer "P2" see Ochsmann (2000) and primers "58SNR1", "58SNR2", "58SNF1", "58SNF2" are after Köhnen (unpubl.). Cycling conditions for amplification consisted of 25 cycles of 96 °C for 60 s, 54 °C for 60 s, 72 °C for 180 s, preceded by an initial denaturation at 96 °C for 90 s and followed by a final extension at 72 °C for 420 s. Samples that failed to amplify for ITS using standard conditions were amplified successfully by adding DMSO to the PCR mix to relax the secondary structure. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen). Cycle sequencing was performed using IRD-labelled primer pairs (MWG-Biotech), the modified "ITS5" (or "ITS5")/"P2" (or "58SNR2") for ITS1 and "58SNF1" (or "ITS3")/the modified "ITS4" (or "ITS4") for ITS2 and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) following the manufacturer's instructions and using the following cycling program: 95 °C for 120 s, 28 cycles of 95 °C for 15 s, 63.4 °C (for ITS1) or 60.4 °C (for ITS2) for 15 s, 70 °C for 17 s. The prepared cycle sequencing products were analysed on a LI-COR DNA sequencer 4000L. Both strands were sequenced to assure accuracy in base calling. All sequences were submitted to EMBL (accession numbers in Table 1).

Sequence alignment. – Sequences were aligned using CLUSTAL W (Thompson & al. 1994, 1997) and alignments adjusted manually. The sequence boundaries between the two spacers and the three coding regions (18S, 5.8S, 25S genes) of nrDNA were determined according to Yokota & al. (1989) and Kim & Jansen (1994). The ends of ITS2 were trimmed (nine bp), since there was no variation. For the Maximum Parsimony analysis, gaps in aligned sequences were treated as missing data, but nine phylogenetically informative indels were coded as binary data and added to the data matrix. In the Bayesian analysis gaps were only treated as missing data.

Maximum Parsimony. – For the Maximum Parsimony (MP) analysis the heuristic search algorithm of PAUP* version 4.0b10 (Swofford 2002) was used with ACCTRAN, MULPARS, TBR branch swapping for 100 random addition sequence replicates. Character states were specified as unordered and unweighted. Support for clades of the strict consensus tree was evaluated using bootstrap (Felsenstein 1985) and decay analyses (Bremer 1988). Bootstrap analysis was performed with 1000 replicates (ACCTRAN, MULPARS, TBR branch swapping) and 10 random addition sequence replicates per bootstrap replicate. Decay analysis was carried out using AutoDecay 3.0 (Eriksson & Wikström 1995) for 100 random addition sequence replicates.

Bayesian Inference. – Bayesian analysis was performed using MrBayes version 3.0b4 (Huelsenbeck & Ronquist 2001, 2004). Modeltest 3.6 (Posada & Crandall 1998) enabled to find the model of DNA substitution (among the 56 models tested) that best fits the data. Modeltest selected TrNef+G by hLRT and GTR+G by Akaike Information Criterion (AIC). The Akaike weights allow a ranking of the models: the larger the AIC difference for a model, the less probable that it is

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Table 1. Sources of the plant material used for SEM study of the pollinarium and for molecular analyses, the latter with the EMBL accession numbers of the obtained sequences. Countries of origin are presented where known.

Species	Source	Pollinarium	ITS1	ITS2
Chrysocycnis schlimii Linden & Rchb. f.	Colombia, O-17251, 124.831, BG Heidelberg	–	AM162235	AM162236
Chrysocycnis schlimii Linden & Rchb. f.	O-17028, BG Heidelberg	+	–	–
Cryptocentrum latifolium Schltr.	Ecuador, 34348, 120.074, BG Heidelberg	+	AM162255	AM162256
Lycaste macrophylla (Poepp. & Endl.) Lindl.	Colombia, O-21506, 120.216, BG Heidelberg	+	AM162259	AM162260
Lycaste skinneri (Bateman ex Lindl.) Lindl.	Mexico, O-21798, 120.245, BG Heidelberg	+	AM162257	AM162258
Maxillaria adendrobium (Rchb. f.) Dressler	Cuba, *Stenzel 757*	–	AM162245	AM162246
Maxillaria alba (Hook.) Lindl.	Cuba, Prov. Holguín, BG Havana	–	AM162219	AM162220
Maxillaria arachnitiflora Ames & C. Schweinf.	Panama, O-18702, BG Jena	+	AM162265	AM162266
Maxillaria camaridii Rchb. f.	89025, BG Jena	+	AM162227	AM162228
Maxillaria coccinea (Jacq.) L. O. Williams	69168, BG Jena	+	AM162247	AM162248
Maxillaria crassifolia (Lindl.) Rchb. f.	Brazil, O-2353, BG Jena	+	AM162239	AM162240
Maxillaria densa Lindl.	07778, BG Jena	+	AM162225	AM162226
Maxillaria friedrichsthalii Rchb. f.	Costa Rica, 08179, BG Jena	+	AM162231	AM162232
Maxillaria nasuta Rchb. f.	Costa Rica, 69176, BG Jena	+	AM162243	AM162244
Maxillaria parkeri Hook.	Ecuador, 69127, BG Jena	+	AM162261	AM162262
Maxillaria parviflora (Poepp. & Endl.) Garay	Cuba, 07732, BG Jena	+	AM1622223	AM1622224
Maxillaria picta Hook.	Brazil, 69118, BG Jena	–	AM162251	AM162252
Maxillaria picta Hook.	08167, *Seidel*	+	–	–
Maxillaria porphyrostele Rchb. f.	Brazil, O-329, BG Jena	–	AM162253	AM162254
Maxillaria porphyrostele Rchb. f.	Brazil, Dathe 6	+	–	–
Maxillaria praestans Rchb. f.	08101, BG Jena	+	AM162229	AM162230
Maxillaria pulla Linden & Rchb. f.	Ecuador, 123.589, BG Heidelberg	+	AM162263	AM162264
Maxillaria rufescens Lindl.	Peru, 25651, BG Jena	+	AM162237	AM162238
Maxillaria sanguinea Rolfe	Costa Rica, O-2303, 121.049, BG Heidelberg	+	AM162215	AM162216
Maxillaria sophronitis (Rchb. f.) Garay	08147, BG Jena	–	AM162249	AM162250
Maxillaria tenuifolia Lindl.	Mexico, 07712, BG Jena	+	AM162271	AM162214
Maxillaria valenzuelana (A. Rich.) Nash	O-10580, 123.012, BG Heidelberg	–	AM162241	AM162242
Maxillaria valenzuelana (A. Rich.) Nash	Cuba, *Dietrich*	+	–	–
Maxillaria variabilis Bateman ex Lindl.	52840b, BG Jena	+	AM162217	AM162218

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Species	Source	Pollinarium	ITS1	ITS2
Maxillaria vitelliniflora Barb. Rodr.	Brazil, 07707, BG Jena	+	AM162221	AM162222
Mormolyca ringens (Lindl.) Schltr.	07769, BG Jena	+	AM162233	AM162234
Trigonidium egertonianum Bateman ex Lindl.	08153, BG Jena	–	AM162267	AM162268
Trigonidium obtusum Lindl.	Brazil, O-17754, 122.077, BG Heidelberg	+	AM162269	AM162270

Table 2. Sequences obtained from EMBL (Whitten & al. 2000). The corresponding taxa are marked with an asterisk "*" in the present work (Fig. 1-2).

Species	Voucher data	Accession number
Cryptocentrum calcaratum (Schltr.) Schltr.	*Whitten* (FLAS)	AF239330
Maxillaria umbratilis L. O. Williams	SEL 1995-0397	AF239331
Maxillaria violaceopunctata Rchb. f.	SEL 1981-2139	AF239332

the best model (Posada & Buckley 2004). Burnham & Anderson (2003) proposed as a rule of thumb that models for which the difference of the AIC value of the respective model and the smallest AIC value among all models $\Delta_i \leq 2$ receive substantial support, whereas models having $\Delta_i \geq 10$ receive no support. In the present data set, the GTR+G has a $\Delta_i = 0$ and the TrNef+G model has a $\Delta_i = 29.3$. Therefore, in the Bayesian analysis only the GTR+G model was used without transferring the parameters estimated by Modeltest as priors to MrBayes, estimating specific substitution rates and the gamma shape parameter as a part of the analysis. Four Metropolis-coupled Markov chain Monte Carlo (MCMC) chains were run for 2 000 000 generations, and one tree for every 100 generations was saved. The first 2000 trees were discarded as burn-in. Based on the remaining 18 001 trees, a consensus tree (allcompat) was calculated.

Split Decomposition. – SplitsTree (Bandelt & Dress 1992a-b) version 4.0 beta 15 (built 1.2.2005) (Huson & Bryant 2006) was used to test how tree-like the given data are. The resulting split decomposition graph indicates in how far tree-like branching pattern is supported by the sequence data or to what extent hybridisation and reticulate evolution may be involved.

Palynological studies. – Air-dried pollinaria were sputtered (gold) and examined with a Stereoscan 360 (Cambridge Instruments) at the Humboldt-Universität zu Berlin, Institute of Physics. The upper half of the pollinium tends to be more stable (Schill & Pfeiffer 1977), so data regarding this portion were used for comparison. From each pollinarium one photo was taken to give a general view of the structure and one or two photos to show the side of the upper pollinium that faces the tapetum. Pollen terminology follows Punt & al. (1994).

Results

Nuclear ribosomal DNA ITS. – The sequences of ITS1 in *Maxillariinae* range in size from 211 *(Cryptocentrum calcaratum*)* to 227 bp *(Maxillaria friedrichsthalii)* and of ITS2 from 239 *(M. variabilis)* to 242 bp. The G + C content varies from 58.90 % *(M. crassifolia)* to 69.20 % *(Trigonidium egertonianum)* in ITS1 and from 63.64 % *(M. crassifolia)* to 73.75 % *(M. parkeri)* in ITS2. The G + C content is stable within clades. The highest G + C content appeared in the *parkeri* clade, the lowest in the *picta* clade. Of the 485 aligned positions (without coded indels),

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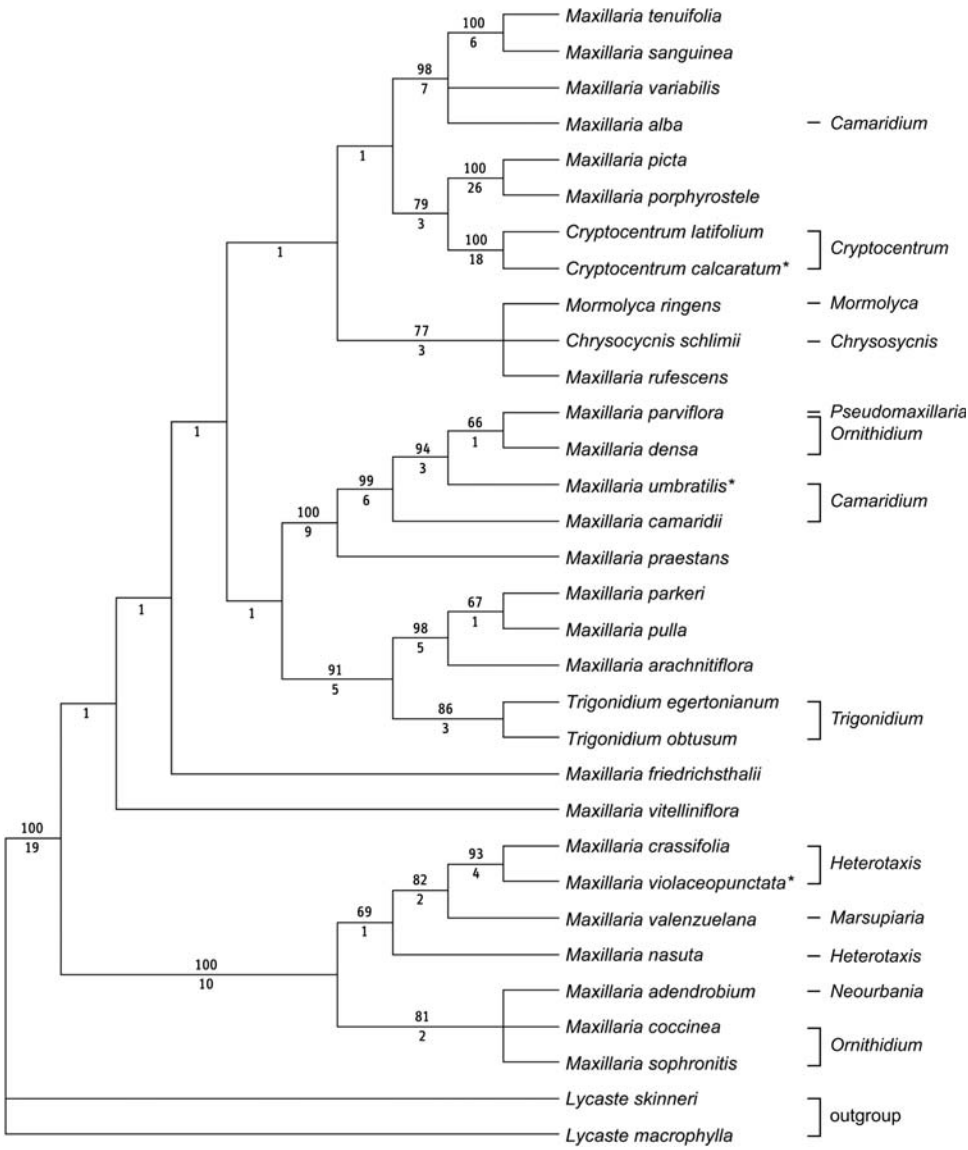


Fig. 1. Strict consensus tree of four equally most parsimonious trees based on ITS sequence data. Gaps are treated as missing data and additionally coded in a binary data matrix. Tree length = 415 steps; CI = 0.6145 (autapomorphies excluded) respectively 0.6916 (autapomorphies included); RI = 0.7515. Bootstrap values > 50 are indicated above, decay values below the branches.

256 characters (53 %) are constant, 80 variable characters (16 %) are parsimony-uninformative and 149 (31 %) are parsimony-informative. The heuristic parsimony search yielded four equally most parsimonious trees with a length of 415 steps, a consistency index (CI) of 0.6145 (autapomorphies excluded) respectively 0.6916 (autapomorphies included) and a retention index (RI) of 0.7515. The strict consensus tree is shown in Fig. 1. The members of the ingroup, the *Maxillariinae,* are monophyletic with high statistic support (100 % bootstrap, 19 steps decay). Within the ingroup, *Maxillaria* is paraphyletic since all examined presently accepted segregates

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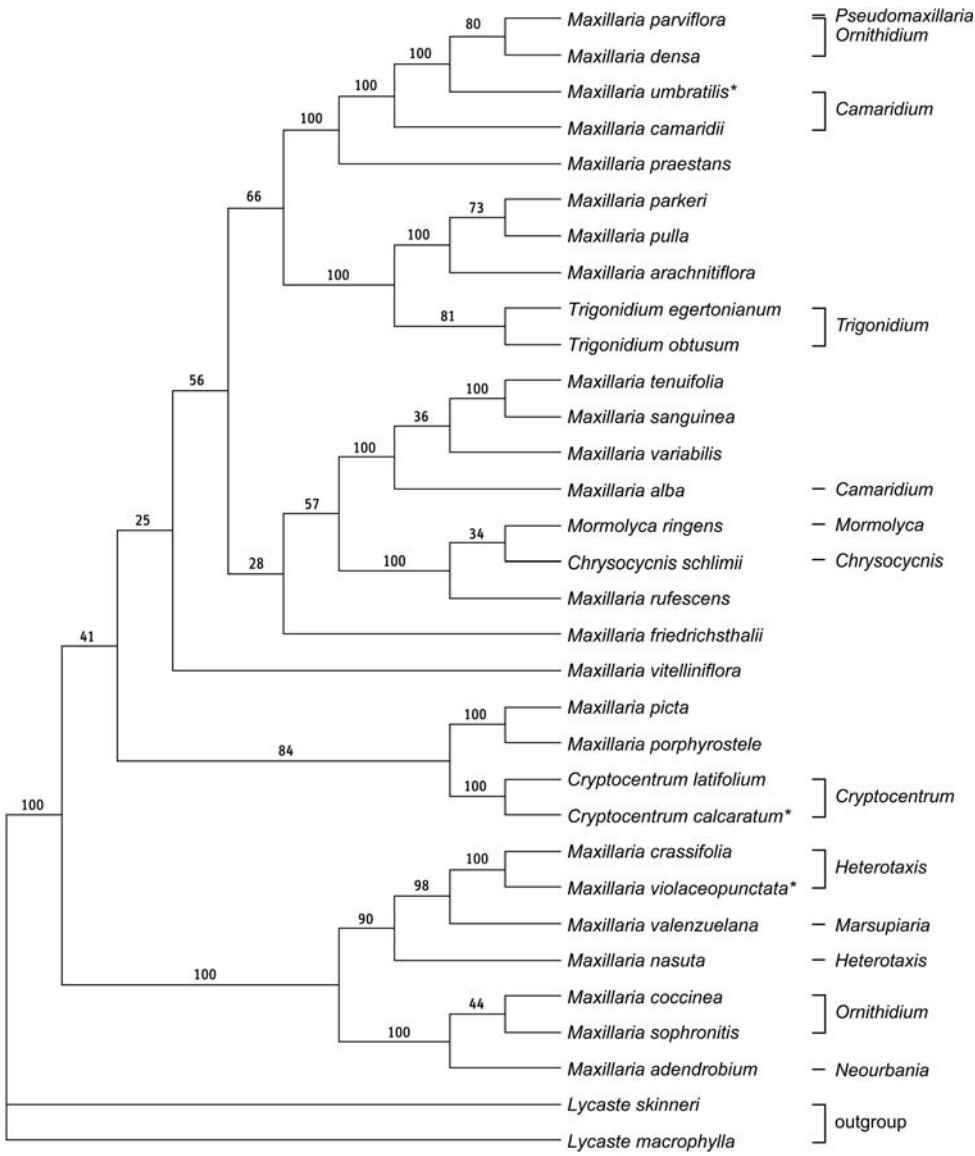


Fig. 2. Consensus tree (allcompat) of 18001 trees from a Bayesian analysis based on ITS sequence data. Gaps are treated as missing data. The GTR+G model of DNA substitution was used. Posterior probabilities (× 100) are indicated above the branches.

(Chrysocycnis, Cryptocentrum, Mormolyca, Trigonidium) as well as the former segregates *Camaridium, Heterotaxis, Marsupiaria, Neourbania, Ornithidium* and *Pseudomaxillaria* are nested within this genus. *Camaridium, Heterotaxis* and *Ornithidium* are also not monophyletic.

There are six clades (terminal nodes) within the ingroup: four show high levels of support (> 90 % bootstrap, 5-10 steps decay), two show moderate support (77 and 79 % bootstrap, 3 steps decay). Since all basal nodes receive only very weak support (1 step decay, < 50 % bootstrap), the relationships among these clades are not resolved.

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The Bayesian analysis yielded the tree topology in Fig. 2. Five of the six moderately to highly supported clades from the MP analysis receive statistical support by a posterior probability of 100 % in this analysis. While the group of *Maxillaria rufescens, Mormolyca ringens* and *Chrysocycnis schlimii* is supported by moderate bootstrap values (77 %), the posterior probability for this clade is significant (100 %). Only the clade with the two members of the *Maxillaria picta* complex and *Cryptocentrum* received no convincing levels of support in both analyses. All basal nodes are weakly supported.

Palynology. – The pollinia vary in shape from spherical (Fig. 3C), ovoid via obovoid, drop-shaped, elongate-clavate (or kidney-shaped) to clavate (Fig. 3A). The length of the larger pollinia ranges from 0.435 mm *(Maxillaria parviflora)* to 1.643 mm *(M. picta)* and they are 0.292 mm *(M. densa)* to 1.143 mm *(M. picta)* wide. Individual pollen grains are often scarcely recognizable within a tetrad. The tetrads vary in size approximately from 16.8 µm *(M. rufescens)* to 34 µm *(M. vitelliniflora).* A species with smaller pollinia has the largest tetrads, whereas a species with larger pollinia possesses the smallest tetrads. The sculpture is more or less rugulate (sometimes gemmate, granulate, fossulate, microfoveolate (Fig. 3D)) to psilate (Fig. 3B). Several tetrads show a harmomegathic effect (collapse of pollen grains caused by air-drying; harmomegathy: accommodation of the pollen grains to different humidity of the atmosphere by contraction or convolution, Wagenitz 1996.)

Discussion

Molecular analysis. – The nuclear ribosomal DNA Internal Transcribed Spacers (ITS) seem to be a useful source of information for understanding phylogenetic relationships within this subtribe. *Maxillariinae* are strongly supported as a monophyletic group but the support depends extremely on the selected outgroup taxa. Previous cladistic analyses of nuclear and chloroplast DNA regions within the tribe *Maxillarieae* (Whitten & al. 2000, Koehler & al. 2002) resulted mostly in poorly resolved trees with low levels of support. Trees calculated from the combined data sets (molecular markers of different DNA regions) showed usually higher resolution and higher support of clades within the ingroup. Outgroup selection for this study was difficult, because all members of the tribe seem to be very closely related. Maximum Parsimony and Bayesian analyses were repeated with representatives of the subtribes *Zygopetalinae* and *Oncidiinae* (tribe *Maxillarieae*) as outgroup. In the Bayesian analyses the ingroup was always supported by a posterior probability of 100 %, regardless of which outgroup was used. Problems occurred in the MP analyses when other taxa than the two *Lycaste* species were chosen as outgroup. In those cases, *Maxillariinae* showed weaker levels of support (between 60 % bootstrap, 1 step decay and 90 % bootstrap, 4 steps decay – depending on indel treatment).

Similar results were obtained by Whitten & al. (2000) for the ITS nrDNA data set. The phenomenon obviously depends on the marker ITS. Our analysis of the data using SplitsTree resulted in a bush-like split decomposition graph, indicating that the sequences could have acquired similar base compositions independently and convergently (see example in Huson 1998). The problem might be caused by random and systematic error or hybridization and further evolutionary events (Bandelt & Dress 1992b). Up to now, there are only few reports and speculations about hybrids (Carnevali & Ramírez de Carnevali 1993, Atwood 2003). It seems that hybridization events, compared with other orchid groups, are not very common within the subtribe *Maxillariinae* (Senghas 1993-94).

However, it should be pointed out that outgroup selection had no significant influence on the statistical support of the clades in the ingroup. The larger matrix slightly increases bootstrap support as well as decay values of the internal nodes, but lowers the CI (because of the higher homoplasy) and the RI. The phylogenetic trees resulting from the MP and the Bayesian analyses differ only slightly, mainly because of their poorly supported basal branches. Possibly the species around *Maxillaria crassifolia* (formerly *Heterotaxis*) are in a basal position in the phylogeny of *Maxillariinae* (see Ojeda & al. 2003, Williams & Whitten 2001).

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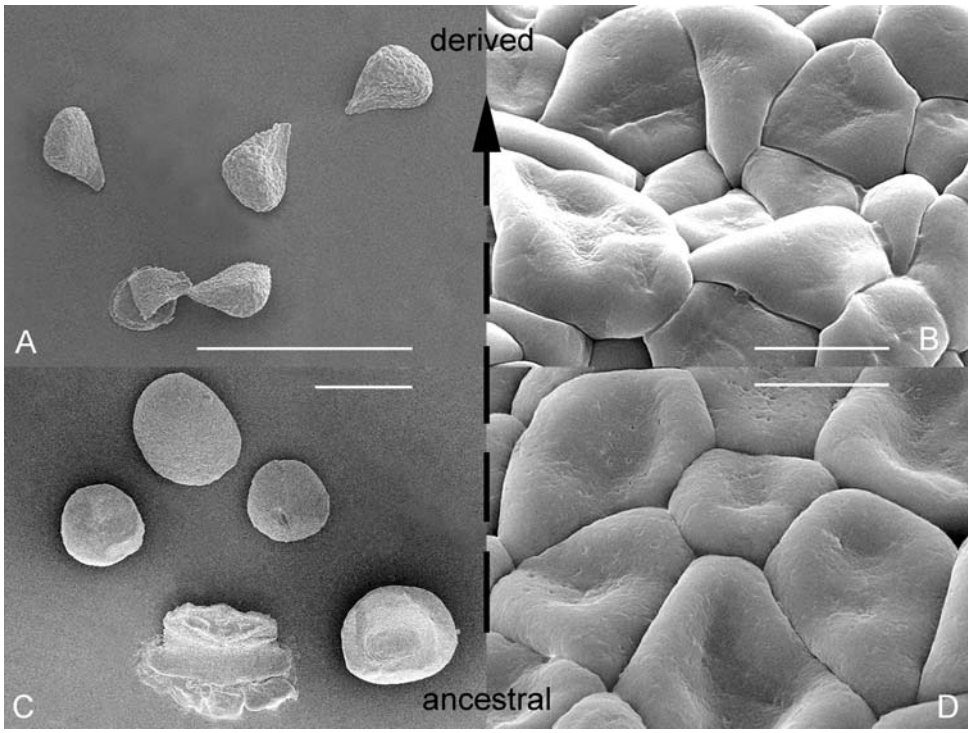


Fig. 3. A-B: *Maxillaria parviflora;* C-D: *M. praestans* – supposed polarity of the developmental lines in the morphology of pollinia and pollinaria in the subtribe *Maxillariinae*. – Scale bars A+C = 1 mm, B+D = 10 µm.

Palynology. – Palynological data are supposed to be ontogenetically and phylogenetically stable (Adams 1958, Schill 1978, Burns-Balogh 1983). As already reported for the exine sculptures of pollinia in advanced *Epidendroideae* by Schill (1975) and Schill & Pfeiffer (1977), the surface is generally more or less smooth, especially in species of *Maxillaria* and *Lycaste*. Our study confirms the observations by Schill (1975), Schill & Pfeiffer (1977) and Dietrich (unpubl. 1989). Pollinarium morphology is stable within the subtribe *Maxillariinae,* but not within generic boundaries (exception: *Ornithidium*). Since Adams (1958) observed variation within species, we examined two individuals each of five species. The pollinaria varied only slightly. Additionally, three individuals of the *Maxillaria rufescens* complex were compared and they varied in nearly all features. The same occurred in the *M. picta* complex: *M. porphyrostele* is clearly distinct from *M. picta* in sculpture.

According to Burns-Balogh & Hesse (1988), the harmomegathic effect is a common feature in taxa with thin exines. Possibly dehydration is needed to prevent insect-mediated self-pollination. Such a mechanism was recently documented in *Trigonidium obtusum* Lindl. (Singer 2002).

In comparison with the sequence data, our palynological observations in *Maxillariinae* suggest three morphological lines: (1) from four greater pollinia in two pairs (Fig. 3C) to four smaller, equal, separate pollinia (Fig. 3A); (2) from spherical (Fig. 3C) to clavate pollinia (Fig. 3A); (3) from rugulate surface (sometimes gemmate, granulate, fossulate, microfoveolate (Fig. 3D)) to psilate surface (Fig. 3B). These lines are not clearly recognizable in the whole tree, but occur in some of the clades. Holttum (1959) supposed a very similar development in pollinia of the *Sarcanthine* orchids, whereas Stenzel (2000) observed contrary trends in *Pleurothallidinae*. Both studies were based on morphological data alone and the polarity of the lines is still unclear (Schill & Pfeiffer 1977). As already proven for other morphological characters, Williams (1970b) and

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Stenzel (2000) suppose parallel or convergent trends in pollinarium/pollinium morphology in the orchid groups they examined.

Phylogeny. – Considering the taxa united in the six clades resulting from our molecular analysis, we notice that some of the species are morphologically very similar to each other (e.g., species of the *Heterotaxis* complex, species around *Maxillaria tenuifolia,* species around *M. rufescens*). Other taxa, in contrast, seem morphologically completely different from their calculated neighbours. This, however, may result from the very small data set, which includes no close relatives for many species.

Regarding growth habit, caespitose species show up in a more or less basal position within a clade (except the *tenuifolia* clade), whereas rhizomatous taxa, with elongate stems or canes (formerly *Camaridium, Ornithidium, Neourbania*), appear several times at terminal nodes. This observation is in contradiction with the supposition that elongate (sometimes branched) stems are primitive within *Orchidaceae* (Dressler & Dodson 1960, Dressler 1993). Interestingly, most of the rhizomatous species were once separated from *Maxillaria,* since commonly caespitose plants have been associated with the genus *Maxillaria* (Senghas 1993-94). However, such a concept of *Maxillaria* is not in line with the typification of the name: only two rhizomatous species, *M. ramosa* Ruiz & Pavón and *M. platypetala* Ruiz & Pavón, are (among the 16 species originally included by Ruiz & Pavón) eligible as lectotype of the name *Maxillaria* (Brieger & Hunt 1969, Senghas 1993-94), of which *M. platypetala* was shown lately to be the only one in full agreement with the protologue (Garay 1997).

The inflorescence of *Ornithidium* with flowers arranged in clusters is supposed to be a primitive feature within this group (Senghas 1993-94, Dietrich unpubl.). Our molecular trees confirm that the sympodial growth habit is plesiomorphic within *Maxillariinae,* as is also assumed for *Orchidaceae* in general (Holttum 1959, Dressler & Dodson 1960, Dressler 1993), whereas the pseudomonopodial growth of *Maxillaria valenzuelana* and some other *Maxillariinae* species is derived (Ojeda & al. 2003).

Singer & Koehler (2004) studied pollinaria and flower rewards in Brazilian *Maxillariinae.* Compared with our molecular data, there is mostly congruence within one clade, so the pollinarium types must have evolved several times independently. The fact that some species of the segregates *Camaridium, Ornithidium* and *Pseudomaxillaria* offer trichomes or nectar as flower reward supports their derived position. Ackerman (1986) regards nectar production as energetically expensive and Dressler (1993) states that nectaries are possibly secondarily derived within *Orchidaceae,* since most primitive orchids lack nectaries. According to Flach & al. (2004), species that offer flower rewards are usually scentless, whereas rewardless flowers are usually scented. Singer & Koehler (2004) found that mainly caespitose species are rewardless. Pseudocopulation as pollination strategy has been suggested to occur in *Chrysocycnis* (rhizomatous) (Van der Pijl & Dodson 1966). *Maxillaria adendrobium* (elongate stems, formerly *Neourbania*) tends to be autogamous on some locations (Dressler 1964, Stenzel pers. comm.). Such unusual pollination syndromes do exist in caespitose species as well (see Singer 2002, Singer & al. 2004, Kirchner 1922), but they could be interpreted as evidence for a derived position.

There are further studies that support at least some of our six clades. For *Maxillaria valenzuelana* (formerly *Marsupiaria*) a closer relationship to *M. crassifolia* (Dressler & Dodson 1960) or the *Heterotaxis* complex (Senghas 1993-94), respectively, based on floral features was already supposed. Ojeda & al. (2003) found that *Heterotaxis* represents a monophyletic group if *Maxillaria valenzuelana* is included and *M. nasuta* (and two other species) are excluded. The close relationship between *Mormolyca ringens* and the *Maxillaria rufescens* complex is supported by anatomy and morphology (Holtzmeier & al. 1998). *Chrysocycnis* shares its outspread flowers with *Mormolyca* (Senghas 1993-94) and some members of the *Maxillaria rufescens* complex (own. obs.). Based on anatomical features, *M. picta* and *M. porphyrostele* form a separate clade within *Maxillaria* (Holtzmeier & al. 1998). On the other hand, there is no real anatomical or morphological evidence for a closer relationship between the *M. picta* complex and *Cryptocentrum,* most of the species within the clades of *M. parkeri* or of *M. densa*.

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Table 3. Frequency of parallelism in 25 *Maxillariinae* species.

Feature	Parallelisms	Feature	Parallelisms
Pseudomonopodial growth	2	Pollinia ± equal in size	4
Rhizomatous growth / canes	4	Pollinia ± clavate	3
Pseudobulbs absent	2	Exine sculpture ± psilate	5
Pseudobulbs 2-foliate	2	Pseudocopulation	2(-3?)
Inflorescence formed in clusters	2	Flower mimicry	2
Spur present	3	Autogamy / Cleistogamy	2
Viscidium not horseshoe-shaped	4	Nectar (flower reward)	3
Stipe elongate	3	Trichomes (flower reward)	3

Incongruence between molecular and non-molecular data can be easily explained with parallelisms. As already observed within other orchid subtribes, e.g., *Laeliinae* (Van den Berg & al. 2000) and *Pleurothallidinae* (Pridgeon & al. 2001), the value of morphological characters in phylogenetic reconstruction of *Maxillariinae* is limited by the high degree of homoplasy. Sixteen features must have evolved at least two times within the limited species sampling of the six clades (Table 3). *Maxillaria friedrichsthalii* and *M. vitelliniflora* were excluded from the statistics, because these taxa could not be assigned to any of the clades.

The great advantage of molecular data is that they are, compared with phenotypical data, less homoplastic, because homologous genes or segments can be examined (Hamby & Zimmer 1992, Hillis & Huelsenbeck 1992). Molecular analyses, therefore can be helpful to reveal parallelisms. However, molecular data are not completely free from parallelisms themselves. Also it cannot, of course, be excluded that due to various biological phenomena the received molecular trees are incongruent with the real phylogeny (Doyle 1992). Moreover, the polarity of the observed morphological lines or growth types remains unresolved.

Our work revealed that *Maxillariinae* show similar conditions as *Pleurothallidinae* (see Pridgeon & al. 2001). As in this subtribe, within *Maxillariinae* probably most of the currently recognized complexes or genera are only useful for identification purposes, but do not represent monophyletic groups (three segregates tested). Further multidisciplinary studies are required to decide if and how *Maxillaria* s.l. can be divided in smaller genera.

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