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# Molecular Phylogeny of the Neotropical Genus *Christensonella* (Orchidaceae, Maxillariinae): Species Delimitation and Insights into Chromosome Evolution

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• *Background and Aims* Species' boundaries applied within *Christensonella* have varied due to the continuous pattern of variation and mosaic distribution of diagnostic characters. The main goals of this study were to revise the species' delimitation and propose a more stable classification for this genus. In order to achieve these aims phylogenetic relationships were inferred using DNA sequence data and cytological diversity within *Christensonella* was examined based on chromosome counts and heterochromatin patterns. The results presented describe sets of diagnostic morphological characters that can be used for species' identification.
• *Methods* Phylogenetic studies were based on sequence data of nuclear and plastid regions, analysed using maximum parsimony and maximum likelihood criteria. Cytogenetic observations of mitotic cells were conducted using CMA and DAPI fluorochromes.
• *Key Results* Six of 21 currently accepted species were recovered. The results also support recognition of the '*C. pumila*' clade as a single species. Molecular phylogenetic relationships within the '*C. acicularis*–*C. madida*' and '*C. ferdinandiana*–*C. neowiedii*' species' complexes were not resolved and require further study. Deeper relationships were incongruent between plastid and nuclear trees, but with no strong bootstrap support for either, except for the position of *C. vernicosa*. Cytogenetic data indicated chromosome numbers of $2n = 36$, 38 and 76, and with substantial variation in the presence and location of CMA/DAPI heterochromatin bands.
• *Conclusions* The recognition of ten species of *Christensonella* is proposed according to the molecular and cytogenetic patterns observed. In addition, diagnostic morphological characters are presented for each recognized species. Banding patterns and chromosome counts suggest the occurrence of centric fusion/fission events, especially for *C. ferdinandiana*. The results suggest that $2n = 36$ karyotypes evolved from $2n = 38$ through descendent dysploidy. Patterns of heterochromatin distribution and other karyotypic data proved to be a valuable source of information to understand evolutionary patterns within Maxillariinae orchids.

**Key words:** Chromosome number, *Christensonella*, Cymbidieae, cytotaxonomy, fluorochrome staining, *Maxillaria*, Maxillariinae, molecular phylogenetics, species delimitation.

## INTRODUCTION

The subtribe Maxillariinae (*sensu* Whitten *et al.*, 2000) comprises a monophyletic group of neotropical orchids with approximately 600–700 species, which are characterized by the presence of a distinct column foot and mentum, four rounded or ovoid pollinia, and a broad, open stigma (Whitten *et al.*, 2000). Although Maxillariinae orchids form a major component of the epiphytic vegetation in the Neotropics, particularly the large and diverse genus *Maxillaria*, they are still taxonomically poorly known. For most species complexes within this genus there is no consensus on how many species should be recognized. In addition, available keys for species' identification are incomplete and subgeneric classifications are highly artificial.

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With the goals of re-evaluating generic boundaries and defining well-supported clades within the subtribe Maxillariinae, Whitten *et al.* (2007) obtained sequence data of multiple regions for 604 specimens belonging to this group. One of the strongly supported clades supported in this study (represented by 61 specimens; Whitten *et al.*, 2007) corresponds to the genus *Christensonella*, also known as the '*Maxillaria madida*' group. This clade was partially recognized by Christenson (2002) as *Maxillaria* section *Urceolatae* (Table 1). Plants within section *Urceolatae* were characterized as densely caespitose small orchids bearing sulcate and cylindrical pseudobulbs with 2–4 needle-like leaves each, subsessile single-flowered inflorescences, flowers with an entire lip, ligulate callus and a long column with unadorned clinadrium and anther, elongated basally in a short foot (Christenson, 2002). Later, Szlachetko *et al.* (2006) transferred all eight species of this section *Urceolatae* to *Christensonella*. Szlachetko *et al.* (2006) also broadened the circumscription

TABLE 1. *Former infrageneric classifications proposed for* Christensonella *(formally regarded as* Maxillaria*)*

| Author | Number of species/infraspecific taxa recognized | Generic classification | Relevant characters |
|---|---|---|---|
| Cogniaux (1904) | 19 taxa | *Maxillaria* | Not presented |
| Hoehne (1953) | 31 taxa | *Maxillaria* | Number and shape of leaves, shape of perianth segments, lip morphology |
| Pabst and Dungs (1977) | 23 spp.; 4 alliances: '*M. madida*', '*M. paulistana*', '*M. pumila*', '*M. subulata*' | *Maxillaria* | Number and shape of leaves |
| Butzin and Senghas (1996) | 9 spp.; 3 groups: 'Nadelblättrige', 'Dickblättrige', 'aufsteigend Kletternde' | *Maxillaria* | Plant architecture and leaf shape |
| Christenson (2002) | 8 spp.; recognized as *Maxillaria* section *Urceolatae* | *Maxillaria* | Plant architecture, leaf shape and reproductive characters |
| Szlachetko *et al.* (2006) | 16 spp. | *Christensonella* | New genus based on Christenson (2002); additional new combinations were not justified |
| Blanco *et al.* (2007) | 4 spp. | *Christensonella* | New combinations based on the results of a broad molecular phylogeny for the subtribe Maxillariinae (Whitten *et al.*, 2007) and according to clear morphological characters |

of Christenson (2002) to include plants with fleshy-to-coriaceous leaves varying from linear to oblong, and conduplicate to cylindrical. Nine species were transfered by Szlachetko *et al.* (2006), resulting in 17 species assigned to *Christensonella* (Table 1). Based on the molecular phylogenetic study of Whitten *et al.* (2007) and according to clear morphological characters, Blanco *et al.* (2007) transferred four additional species to *Christensonella* (Table 1), increasing the total number of species in this genus to 21. Species belonging to *Christensonella* can also be easily recognized by their whitish-to-yellow or red-to-maroon coloured flowers, generally with a shiny spot in the midlobe lip and, commonly, by the roots with annular expansions of the velamen (Blanco *et al.*, 2007). Most species are restricted to south-eastern South America, ranging from the south of Bahia State, in Brazil, to Misiones, in Argentina. A few species, however, are restricted to Central America and western South America, occurring from Bolivia up to southern Mexico.

Although *Christensonella* is easily distinguishable from other Maxillariinae, species' identification within it has been very challenging. Species' delimitation within this genus, especially the south-eastern Brazilian species, has varied widely among taxonomic treatments, in part due to the mosaic distribution of diagnostic characters among taxa, but also because of the continuously variable nature of morphological traits (Cogniaux, 1904; Hoehne, 1953; Pabst and Dungs, 1977; Butzin and Senghas, 1996; Table 1).

In the first taxonomic treatment of the genus *Maxillaria* in Brazil, Cogniaux (1904) recognized 19 taxa (17 species and two new varieties), which agree with the morphological circumscription of *Christensonella*. Later, in several publications concerning the taxonomy of Brazilian species of *Maxillaria*, Hoehne (1947, 1952, 1953) described several new taxa, recognizing a total of 31 that are morphologically consistent with *Christensonella* (Hoehne, 1953). The diagnostic characters regarded as important by Hoehne (1953) to identify species within this group were number and shape of leaves, shape of perianth segments, and lip morphology (Table 1). Species' delimitation in Hoehne's work was clearly influenced by the typological species' concept, with many taxa being recognized as a function of high polymorphism of vegetative and flower characters among populations (Hoehne, 1953).

In their classification of Brazilian orchids, Pabst and Dungs (1977) divided the species of *Maxillaria* into several alliances, primarily according to vegetative traits. They grouped all the Brazilian species currently recognized as *Christensonella* into four different alliances based upon the number and shape of leaves (Pabst and Dungs 1977). The only exception was *Maxillaria uncata* [= *Christensonella uncata*], which was assigned to a distinct alliance based on the presence of a long rhizome and an undivided lip (Pabst and Dungs, 1977).

In the last edition of Schlechter's *Die Orchideen* (Butzin and Senghas, 1996), nine species of *Maxillaria*, currently recognized as belonging to *Christensonella* (Szlachetko *et al.*, 2006), were divided into three main groups, according to leaf shape and plant architecture. The placement of the species *M. madida* [= *C. madida*] in two different groups *per se* reflects the high infra-specific polymorphism of vegetative characters used for assigning group membership and the difficulty of classifying taxa within this clade based solely on morphological data. Interestingly, Schlechter was the only author, apart from Szlachetko *et al.* (2006), to consider *C. uncata* (which occurs from north-western South America to Mexico) and *C. nardoides* (from Peru) to belong to this clade (mostly restricted to south-east South America) based on the presence of fleshy leaves, erect pseudobulbs with brownish papery bracts, and medium-size, partially closed flowers, varying from yellowish to brownish.

The available taxonomic treatments fail to provide clear boundaries for species within *Christensonella*, possibly because of the continuous variable nature of morphological characters among current recognized species, especially plant architecture, leaf shape and lip morphology (Cogniaux, 1904; Hoehne, 1953; Pabst and Dungs, 1977; Butzin and Senghas, 1996). Some species belonging to this group also present extremely high phenotypic plasticity of vegetative characters, as observed for some cultivated

specimens, probably related to different light conditions and humidity levels (S. Koehler, pers. obs.). Such extreme morphological variation within and among putative species and infra-specific taxa resulted in morphologically undiagnosable species.

The main goals of this study were to understand patterns of diversification within the genus *Christensonella* in order to revise species' delimitation and provide a more stable classification for this group. To achieve such aims, we (1) inferred phylogenetic relationships within *Christensonella* based on sequence data from the plastid *trnL* intron and *trnL–F* intergenic spacer, the plastid *matK* gene, the *atpB-rbcL* spacer, and nuclear ribosomal internal transcribed spacers [ITS1-2] DNA regions; and (2) described sets of diagnostic morphological characters that can be used for species' identification according to the molecular patterns here obtained. Additionally, cytological diversity within *Christensonella* was also examined based on chromosome counts and heterochromatin patterns. Despite being one of the largest subtribes of the Orchidaceae, cytogenetic data on Maxillariinae species such as *Christensonella* are scarce and chromosome evolution within this genus remains poorly understood (Cabral *et al.*, 2006; Whitten *et al.*, 2007). Cytogenetic data are an important source of information that might help phylogenetic studies (Dobigny *et al.*, 2004). Both the variation in chromosome numbers and the study of patterns of heterochromatin distribution have proved to be valuable tools in species' and generic delimitation (Brandham, 1999; Guerra, 2000; Brasileiro-Vidal *et al.*, 2007), including within the subtribe Maxillariinae (Cabral *et al.*, 2006).

## MATERIALS AND METHODS

Since the monophyly of *Christensonella* was confirmed by Whitten *et al.* (2007), only two outgroup species, *Maxillaria crocea* and *M. ochroleuca*, were included in this study for tree rooting purposes (although the sister group to *Christensonella* is not resolved; Whitten *et al.*, 2007). Sampling included 48 specimens of the ingroup with at least two individuals of all species except for *C. cogniauxiana* and *Christensonella* sp. *Whitten 2310* (an undescribed species). The study was based on a total of 182 sequences, 127 of which were already published as part of a study of generic delimitation of the subtribe Maxillariinae (Whitten *et al.*, 2007; Table 2). Sequence data was added from the plastid *trnL* intron and *trnL–F* intergenic spacer and complementary data of the other three regions was obtained for additional specimens (Table 2). All samples were vouchered as herbarium specimens, including the ones used for cytogenetic studies (Tables 2 and 3). Samples *Koehler 73*, *Koehler 79*, *Koehler 91 and Koehler 240* were excluded from the ITS matrix, as were samples *Koehler 173*, *Koehler 243 and Whitten 951* from the plastid matrix due to the poor quality of the data.

We followed the species' criteria of Hoehne (1953) for preliminary identification and discussion of species limits within *Christensonella*, since it comprises the most recent taxonomic treatment available for the majority of species belonging to this clade. Three species recognized by Hoehne (1953), *Maxillaria heterophylla*, *M. mosenii* and *M. plebeja*, have not been transferred to *Christensonella* yet and therefore are treated here as *Maxillaria*. We based our re-evaluation of species' limits on well-supported monophyletic groups of specimens that could be identified by sets of morphological and/or cytological characters.

### *Laboratory protocols*

DNA was extracted from fresh plant tissues (leaves and flowers) from plants available in cultivation according to Doyle and Doyle (1987) and scaled down to 1-mL extraction volumes following the protocol described by Whitten *et al.* (2000), except that all total DNA extracts were purified with QIAquick columns (Qiagen Inc.) prior to amplification. Amplification was performed in 25–50-mL reactions, with 2·5 mM $MgCl_2$, and Sigma buffers (Sigma Inc.), 0·2–0·4 mM of each primer, 1 U of *Taq* polymerase and 50–300 ng of template. In all ITS amplifications, betaine (Sigma Inc.) was added (1·0 mM final concentration) to the PCR mix to relax secondary structure. Amplification and sequencing primers used for ITS and *trnL–F* regions are those of Sun *et al.* (1994) and Taberlet *et al.* (1991), respectively. Some amplifications for *trnL–F* using primers C and F produced multiple bands; for these samples, the region was amplified in two separate reactions using primer pairs C + D and E + F. For the amplification of *matK* and *atpB–rbcL* spacer regions, primers specifically designed for Maxillariinae orchids were used (Whitten *et al.*, 2007). The *matK* + *trnK* intron region was usually amplified as a single piece, using the primers -19F (Goldman *et al.*, 2001) and *trnK*2R (Johnson and Soltis, 1994); primers 308F and 1100F (Whitten *et al.*, 2007) were used as additional internal sequencing primers. Some taxa were amplified using the primers 56F and 1520R that yielded a shorter but nearly complete portion of *matK* (Whitten *et al.*, 2007). The *atpB–rbcL* intergenic spacer was amplified with the primers Max F and Max R (Whitten *et al.*, 2007). Protocols for the amplification reactions were as follows. ITS: 10 min initial denaturation at 99 °C, 30 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s, 72 °C extension for 1 min; *trnL-F*: 32 cycles of 94 °C denaturation for 30 s, 61 °C annealing for 30 s, 72 °C extension for 75 s; *matK* and *atpB–rbcL* spacer: 33 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s, 72 °C extension for 2 min. Amplified products were purified with QIAquick PCR cleaning column and filtration kit (Qiagen Inc.) and directly sequenced on Applied Biosystems, Inc (ABI) 373/377 or 3100/3500 automated sequencers using standard dye-terminator according to the manufacturer's protocols, except that cycle sequencing reactions were scaled down to 5 mL. Both strands were sequenced to assure accuracy in base calling.

### *Data analysis*

*Alignment.* The software packages 'Sequence Navigator™', 'Autoassembler™' (ABI) and 'Sequencher™' (Gene Codes Corporation) were used to edit and assemble complementary

TABLE 2. *DNA vouchers and GenBank accession numbers for specimens used in this study. DQ, Sequences previously published in Whitten* et al. *(2007); EU, additional sequences obtained in this study*

| Taxon | Collector | Country, State | Locality | Herbarium | nrITS | *matK* | *atpB–rbcL* spacer | *trnL–trnF* |
|---|---|---|---|---|---|---|---|---|
| *Christensonella acicularis* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0115* | Brazil: BA | ex *E.F. Silva sn* | SP | DQ210142 | DQ210673 | DQ209452 | EU099775 |
| *Christensonella acicularis* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0237* | Brazil, RJ | Nova Friburgo | UEC | DQ210161 | DQ210693 | DQ209469 | EU099776 |
| *Christensonella acicularis* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0352* | Brazil | Cultivated | UEC | DQ210196 | DQ210726 | DQ209503 | EU099780 |
| *Christensonella acicularis* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0371* | Brazil, RJ | Nova Friburgo, 600 m | ESA | DQ210204 | DQ210734 | DQ209511 | n/a |
| *Christensonella acicularis* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 1994* | Brazil | Cultivated | FLAS | DQ210301 | DQ210800 | n/a | EU099786 |
| *Christensonella echinophyta* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0353* | Brazil | Orquidário Bela Vista | UEC | DQ210197 | DQ210727 | DQ209504 | EU099781 |
| *Christensonella echinophyta* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 0951* | Brazil | Cultivated | UEC | n/a | EU101461 | n/a | n/a |
| *Christensonella echinophyta* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 1056* | Brazil | Cultivated | FLAS | DQ210250 | DQ210765 | DQ209544 | EU099785 |
| *Christensonella ferdinandiana* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0089* | Brazil, SC | approx. Orleans, *Bicalho & Targa sn* | SP | DQ210129 | DQ210660 | DQ209440 | EU099764 |
| *Christensonella ferdinandiana* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0109* | Brazil, MG | Camanduacaia | SP | DQ210139 | DQ210670 | DQ209449 | EU099772 |
| *Christensonella juergensii* (Schltr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0069* | Brazil, SP | 17 km from Itaperai, São Francisco farm, *Brolio sn* | SP | DQ210120 | DQ210651 | DQ209431 | EU099759 |
| *Christensonella juergensii* (Schltr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0079* | Brazil | Cultivated | SP | DQ210124 | DQ210655 | DQ209435 | n/a |
| *Christensonella juergensii* (Schltr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0111* | Brazil | Cultivated | SP | DQ210140 | DQ210671 | DQ209450 | EU099773 |
| *Christensonella madida* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0065* | Brazil, SP | Road Cunha-Parati, Pico da Serra 1500 m | SP | DQ210119 | DQ210650 | DQ209430 | EU099758 |
| *Christensonella madida* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0081* | Brazil, SP | Reserva Biológica, *VL Gil*, *M Salcane*, *P Brolio 23* | SP | DQ210125 | DQ210656 | DQ209436 | EU099761 |
| *Christensonella madida* (Lindl) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0107* | Brazil, RJ | Petrópolis, Correas | SP | DQ210138 | DQ210669 | DQ209448 | EU099771 |
| *Christensonella minuta* (Cogn.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0097* | Brazil | Cultivated | SP | DQ210133 | DQ210664 | DQ209444 | EU099766 |
| *Christensonella minuta* (Cogn.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0253* | Brazil, ES | Sooretama, *Koehler & Singer sn* | UEC | DQ210166 | DQ210696 | DQ209474 | EU099778 |
| *Christensonella nardoides* (Kraenzl.) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 2359* | Ecuador | Cultivated Ecuagenera | FLAS | DQ210335 | DQ210833 | n/a | n/a |
| *Christensonella nardoides* (Kraenzl.) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 2502* | Ecuador | Cultivated Ecuagenera | FLAS | DQ210403 | DQ210890 | DQ209688 | EU099791 |

*Continued*

TABLE 2. *Continued*

| Taxon | Collector | Country, State | Locality | Herbarium | nrITS | *matK* | *atpB–rbcL* spacer | *trnL–trnF* |
|---|---|---|---|---|---|---|---|---|
| *Christensonella neowiedii* (Rchb.f.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0073* | Brazil, SP | Road Cunha-Parati, Pico da Serra 1500 m | SP | DQ210122 | DQ210653 | DQ209433 | n/a |
| *Christensonella neowiedii* (Rchb.f.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0091* | Brazi, RJ | Nova Friburgo, Lumiar | SP | DQ210130 | DQ210661 | DQ209441 | n/a |
| *Christensonella pacholskii* (Christenson) S. Koehler | *Whitten 2393* | Ecuador | Cultivated Ecuagenera | FLAS | DQ210355 | DQ210851 | DQ209642 | EU099788 |
| *Christensonella pacholskii* (Christenson) S. Koehler | *Whitten 2464* | Ecuador | Cultivated Ecuagenera | FLAS | DQ210382 | DQ210873 | DQ209668 | EU099790 |
| *Christensonella pachyphylla* (Schltr. ex Hoehne) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0105* | Brazil, SC | São Lourençinho river | SP | DQ210137 | DQ210668 | EU101458 | EU099770 |
| *Christensonella pachyphylla* (Schltr. ex Hoehne) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0369* | Brazil | Cultivated | ESA | DQ210203 | DQ210733 | DQ209510 | n/a |
| *Christensonella pumila* (Hook.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0094* | Brazil | Cultivated | SP | DQ210131 | DQ210662 | DQ209442 | EU099765 |
| *Christensonella pumila* (Hook.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0101* | Brazil, SP | Peruíbe, matas do clube de caça e pesca Garaú | SP | DQ210135 | DQ210666 | DQ209446 | EU099768 |
| *Christensonella pumila* (Hook.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0355* | Brazil, SP | Campos do Jordão | UEC | DQ210198 | DQ210728 | DQ209505 | EU099782 |
| *Christensonella uncata* (Lindl.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0075* | Brazil | Cultivated | SP | DQ210123 | DQ210654 | DQ209434 | n/a |
| *Christensonella uncata* (Lindl.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0359* | Brazil | Orquidário Bela Vista | UEC | DQ210199 | DQ210729 | DQ209506 | n/a |
| *Christensonella uncata* (Lindl.) Szlach., Mytnik, Górniak & Śmiszek | *Whitten 2394* | Ecuador | Cultivated Ecuagenera | FLAS | DQ210356 | DQ210852 | DQ209643 | EU099789 |
| *Christensonella vernicosa* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0099* | Brazil, MG | Caldas | SP | DQ210134 | DQ210665 | DQ209445 | EU099767 |
| *Christensonella vernicosa* (Barb.Rodr.) Szlach., Mytnik, Górniak & Śmiszek | *Koehler 0103* | Brazil, BA | Castro Alves, Garrão José Rodrigues farm | SP | DQ210136 | DQ210667 | DQ209447 | EU099769 |
| *Christensonella* sp. | *Whitten 2310* | Peru | Cultivated | FLAS | DQ210317 | DQ210816 | DQ209605 | EU099787 |
| *Maxillaria plebeja* Rchb.f. | *Koehler 0085* | Brazil, SP | Bertioga, Estação Ecológica Boracéia, *G. Neto sn* | SP | DQ210127 | DQ210658 | DQ209438 | EU099763 |
| *Maxillaria plebeja* Rchb.f. | *Koehler 1653* | Brazil, MG | Lagoa Grande, Belo Horizonte-Ouro Preto road | SP | DQ210207 | DQ210737 | DQ209514 | EU099783 |
| *Maxillaria crocea* Poepp & Endl. | *Koehler 0005* | Brazil | Cultivated | UEC | EU101459 | EU101460 | EU101457 | EU099756 |
| *Maxillaria heterophylla* var. *acicularifolia* Hoehne | *Koehler 0095* | Brazil, SP | 17 km from Itaperai, São Francisco farm, *Brolio sn* | SP | DQ210132 | DQ210663 | DQ209443 | n/a |
| *Maxillaria heterophylla* var. *acicularifolia* Hoehne | *Koehler 1706* | Brazil, SP | Cotia, reserva florestal Morro Grande | SP | DQ210208 | DQ210738 | DQ209515 | EU099784 |
| *Maxillaria heterophylla* var. *intermedia* Hoehne | *Koehler 0240* | Brazil | Orquidário Bela Vista | UEC | DQ210162 | n/a | DQ209470 | n/a |
| *Maxillaria heterophylla* var. *magnifolia* Hoehne | *Koehler 0245* | Brazil, RJ | Nova Friburgo, Macaé de Cima, *Koehler & Pinheiro sn* | UEC | DQ210165 | DQ210695 | DQ209473 | EU099777 |
| *Maxillaria heterophylla* var. *pygmaea* Hoehne | *Koehler 0113* | Brazil, SP | Cotia | SP | DQ210141 | DQ210672 | DQ209451 | EU099774 |
| *Maxillaria heterophylla* var. *pygmaea* Hoehne | *Koehler 0278* | Brazil, MG | Serra do Cipó | ESA | DQ210174 | DQ210704 | DQ209481 | n/a |

*Continued*

TABLE 2. *Continued*

| Taxon | Collector | Country, State | Locality | Herbarium | nrITS | *matK* | *atpB–rbcL* spacer | *trnL–trnF* |
|---|---|---|---|---|---|---|---|---|
| *Maxillaria heterophylla* var. *pygmaea* Hoehne | *Koehler 0292* | Brazil, SP | Campos do Jordão, Parque Florestal, 1700 m, *P. S. Martins sn* | ESA | DQ210176 | DQ210706 | DQ209483 | n/a |
| *Maxillaria mosenii* var. *echinochila* Hoehne | *Koehler 0087* | Brazil, MG | Santana do Riacho, Serra do Cipó (Palácio), *Bicalho sn* | SP | DQ210128 | DQ210659 | DQ209439 | n/a |
| *Maxillaria mosenii* var. *echinochila* Hoehne | *Koehler 0294* | Brazil, MG | Presidente Jucelino, *Bicalho sn* | ESA | DQ210177 | DQ210707 | DQ209484 | EU099779 |
| *Maxillaria mosenii* var. *hatschbachii* Hoehne | *Koehler 0071* | Brazil, SP | Cananéia, I. Cardoso, *T. Breier 273* | UEC | DQ210121 | DQ210652 | DQ209432 | EU099760 |
| *Maxillaria mosenii* var. *hatschbachii* Hoehne | *Koehler 0083* | Brazil, SP | Santo André, Paranapiacaba, Reserva Biológica, *Barros sn* | SP | DQ210126 | DQ210657 | DQ209437 | EU099762 |
| *Maxillaria ochroleuca* Lodd. ex Lindl. | *Koehler 0011* | Brazil, SP | Cananéia,I. Cardoso, *T. Breier sn* | UEC | DQ210105 | DQ210636 | DQ209417 | EU099757 |

TABLE 3. *Species of* Christensonella/Maxillaria *sampled for cytogenetic studies, with respective locality, voucher number, diploid chromosome number, predominant type of chromosomes (meta = metacentric, sub = sub-metacentric, acro = acrocentric) and the distribution patterns (for the diploid complement) of DAPI$^+$ and CMA$^+$ bands. Note that DAPI$^+$ bands were always proximal to the centromere and the type of the banded chromosome is indicated as 'm' for metacentric, 'sm' for sub-metacentric and 'a' for acrocentric. For* C. ferdinandiana*, with four DAPI$^+$ bands, only one chromosome pair was banded, showing two proximal bands for each chromosome (see Fig. 3A). The CMA$^+$ bands were always in the long arm of acrocentric chromosomes and the position of the band is indicated as 't' for terminal, 'st' for sub-terminal, 'i' for interstitial*

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

and overlapping sequences. Each individual base position was examined for agreement of the two strands. DNA sequences were aligned manually using Se-Al (Rambaut, 2002), and gaps were coded as missing values. Terminal priming regions were excluded, as were regions where alignment was ambiguous or where extensive length variation occurred. All the aligned matrices are available upon request from S. Koehler.

*Data exploration.* The null hypothesis of base frequency stationary among sequences was evaluated using the chi-square heterogeneity test as implemented in PAUP* 4·0b10 (Swofford, 2000). The g1 statistic was used to determine if the phylogenetic signal was significantly non-random (Hillis and Huelsenbeck, 1992). The left skew of tree distributions was obtained in PAUP* based on 10 000 randomly generated trees. Possible incongruence between nuclear and chloroplast genomes was assessed with the incongruence length difference (ILD) test (Farris *et al.*, 1994), implemented in PAUP* as the partition homogeneity test using 1000 replicates and excluding uninformative characters to avoid over-estimation of the amount of incongruence (Lee, 2001).

*Phylogenetic analyses.* Phylogenetic analyses were initially conducted with a heuristic search under the maximum parsimony (MP) criterion of Fitch (unordered characters, equal weights to all changes; Fitch, 1971), excluding uninformative characters, and with ACCTRAN optimization. The search strategy for all data sets used 10 000 addition sequence replicates by stepwise addition holding ten trees per replicate, TBR branch swapping on best trees, MULTREES on, saving no more than ten optimal trees per replicate. To assess support for internal clades we performed 1000 bootstrap pseudo-replicates (Felsenstein, 1985) of ten addition sequence replicates by stepwise addition holding one tree per replicate. The categories of bootstrap support considered in this study were: unsupported (<50 %); weak (50–74 %); moderate (75–84 %); strong (85–100 %) (Whitten *et al.*, 2000). Since simulation experiments have shown that high levels of homoplasy can decrease the accuracy of phylogenetic inference under the parsimony criterion (Huelsenbeck and Hillis, 1993), we also employed the successive weighting strategy (SW) for maximum parsimony analyses (Farris, 1969; Carpenter, 1994). Optimization of successive weighting analyses was carried out considering 1000 addition sequence replicates and SPR branch swapping with characters being reweighed according to the rescaled consistency index until tree scores were not improved. Then, a final analysis considering the same search strategy applied to the unweighted data was conducted (10 000 addition sequence replicates, TBR branch swapping).

For maximum likelihood analyses (ML), alternative nested models of DNA sequence evolution were first evaluated with likelihood ratio tests as implemented in MODELTEST 3·7 (Posada and Crandall, 1998; $\alpha = 0{\cdot}01$). The best-fit model of DNA sequence evolution with its estimated parameters was then input into detailed maximum-likelihood tree searches performed in PAUP*. Starting trees were obtained using ten addition sequence replicates by stepwise addition holding 1 tree per replicate, with further SPR branch swapping. Starting branch lengths were obtained using the Rogers–Swofford approximation method with the branch-length optimization of Newton–Raphson. Confidence of the ML trees obtained was assessed by bootstrap analyses based on 100 pseudo-replicates using the fast reduced search option in PAUP*.

*Cytogenetic studies*

Root tips were collected and pretreated in 0·002 M 8-hydroxyquinoline for 20 h at 8 °C. Samples were then fixed in Carnoy's solution (ethanol/glacial acetic acid, 3:1, v/v) for 2 h at room temperature and stored at −20 °C. Root tips were digested with 2 % cellulase and 20 % pectinase (both w/v) for 90 min at 37 °C. The meristem was subsequently isolated and squashed in 45 % acetic acid. After removing the cover-slip the slides were air-dried and aged for 3 d at room temperature.

The aged slides were double stained according to Schweizer and Ambros (1994) with CMA (0·5 mg $mL^{-1}$, 1 h) and DAPI (2 μg $mL^{-1}$, 30 min), and mounted in McIlvaine's (pH 7·0) buffer–glycerol (1 : 1, v/v) containing 2·5 mM $MgCl_2$. After being aged at room temperature for at least 3 d for fluorochrome stabilization, the best slides were analysed using a Leica DMLB microscope and the cell images were captured with a COHU digital camera using the QFISH software (Leica).

## RESULTS

*Phylogenetic analyses*

The test for phylogenetic signal based on random-tree distributions showed that all data sets contain significant phylogenetic information (g1 values: ITS = −0·40, plastid = −0·55). We were unable to detect any significant heterogeneity in base frequencies among taxa using the chi-square heterogeneity test for the ITS data set ($P = 0{\cdot}99$). However, the chi-square test rejected the hypothesis of base frequency homogeneity for the plastid data set ($P = 0{\cdot}004$). The null hypothesis of congruence between the nuclear and plastid data sets was strongly rejected by the ILD test ($P = 0{\cdot}001$). As visual inspection of nuclear and plastid topologies also indicated they are discordant, we chose not to discuss the results based on a single combined analysis (but see Discussion, below). Tree statistics of MP and SW analyses are summarized in Table 4. Bootstrap analyses were conducted only for unweighted data.

*ITS.* The MP and ML statistics for the ITS analyses are given in Tables 4 and 5, respectively. Most traditionally recognized species (Hoehne, 1953; Pabst and Dungs, 1977) are not monophyletic according to the ITS tree (Fig. 1). Five morphologically distinct species are strongly supported as monophyletic: *Christensonella echinophyta*, *C. nardoides*, *C. pachyphylla*, *C. uncata* and *C. vernicosa*. Two species' complexes, composed of species with extremely similar morphology, also emerged as monophyletic groups from this analysis: the '*Christensonella acicularis–C. madida–Maxillaria mosenii*' clade and the '*Christensonella minuta–C. pumila–Maxillaria plebeja*' clade: these are designated

TABLE 4. *Statistics from phylogenetic analyses performed under the maximum parsimony criterion*

| Data set | No. of ingroup taxa | Chi-square homogeneity test results | g1 statistics | Total no. of characters (informative) | MPT* | Length* | CI* | RI* | RC |
|---|---|---|---|---|---|---|---|---|---|
| ITS | 49 | 91·19 (d.f. = 150, $P = 0·99$) | −0·40 | 748 (14·4 %) | 1440 (260) | 166 (127·3) | 0·80 (0·90) | 0·95 (0·98) | 0·88 |
| plastid | 48 | 189·96 (d.f. = 141, $P = 0·004$) | −0·55 | 4235 (6·3 %) | 39 366 (2257) | 454 (232·9) | 0·65 (0·88) | 0·87 (0·97) | 0·85 |

* Results under successive weighting strategy in brackets.

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

| Data set | No. of ingroup taxa | Selected model | Nucleotide frequencies | Shape parameter ($\alpha$-value) of gamma-distributed rate variation across sites | Pinvar | −lnL value |
|---|---|---|---|---|---|---|
| ITS | 49 | General Time Reversible | A = 0·21, C = 0·28, G = 0·32, T = 0·19 | 0·47 | n/a | 2528·53 |
| Plastid | 46 | F81 + G + I | A = 0·32, C = 0·15, G = 0·14, T = 0·39 | 0·90 | 0·61 | 10986·45 |

here as '*C. acicularis*–*C. madida*' (Fig. 1A) and '*C. pumila*' (Fig. 1D), respectively. The clade *Christensonella cogniauxiana*–*C. ferdinandiana*–*C. juergensii*–*Maxillaria heterophylla*–*C. neowiedii* (= '*C. ferdinandiana*–*C. neowiedii*' clade, Fig. 1C), comprises two morphologically distinct groups, the species *C. ferdinandiana* and the '*C. cogniauxiana*–*C. juergensii*–*M. heterophylla*–*C. neowiedii*' group. The currently accepted species *C. pacholskii* also appears as a monophyletic group in the ITS analysis, but with weak bootstrap support (Fig. 1I). Other strongly supported clades obtained from both MP and ML analyses of ITS data were (1) the '*C. acicularis*–*C. madida*' clade + *C. nardoides* + *Christensonella* sp; (2) *C. uncata* + *C. vernicosa* + *C. pacholskii*; and (3) *C. echinophyta* + *C. pachyphylla* + the '*C. pumila*' clade + the '*C. ferdinandiana*–*C. neowiedii*' clade (Fig. 1). Two clades were only recovered in the ML and SW analyses: '*C. acicularis*−*C. madida*' clade + *C. nardoides* and (*C. pachyphylla*, '*C. ferdinandiana*−*C. neowiedii*' clade, *C. echinophyta*, '*C. pumila*' clade) + (*C. uncata, C. vernicosa, C. pacholskii*)) (Fig. 1).

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

Despite the many terminal clades in common with the ITS data set, deeper nodes in the ML, MP and SW plastid trees were incongruent with those based on ITS data (Figs 1 and 2). The plastid trees did not support the clade '*C. uncata* + *C. vernicosa* + *C. pacholskii*' as sister to the clade '*C. pumila*' + '*C. juergensii*–*C. ferdinandiana*' + *C. echinophyta* + *C. pachyphylla*. Instead, the latter is indicated as sister to the '*C. acicularis*–*C. madida'* clade, with *C. vernicosa* embedded in it, although none of these alternatives received bootstrap support greater than 50 % (nodes collapsed). The plastid tree also supports *C. nardoides* as sister to *Christensonella* sp., whereas ITS data supports the former as sister to the '*C. acicularis*–*C. madida*' clade. However, none of these clades received bootstrap support higher than 50 %: MP analyses of plastid data did not even support *Christensonella* sp. and *C. nardoides* as sister to the '*C. acicularis*–*C. madida*' clade (Fig. 2).

### *Cytogenetic studies*

Cytogenetic data for the species analysed in the present study are summarized in Table 3. Three different chromosome numbers were found: $2n = 36$ (*C. ferdinandiana*, *C. heterophylla*, *C. pachyphylla*, *C. pumila*); $2n = 38$ (*C. acicularis*, *C. madida*, *M. mosenii*), and $2n = 76$ (*M. mosenii* var. *echinochila*) (Figs 3, 4). All species studied revealed symmetric karyotypes with predominance of acrocentric chromosomes, except for the species with $2n = 36$, which also showed at least two metacentric or submetacentric chromosomes (Fig. 3).

$CMA^+$ bands varied in number and were mainly terminal or sub-terminal on the long arm of acrocentric chromosome pairs, while $DAPI^+$ bands varied in number but were always proximal to the centromere (Table 3). Heteromorphism of $CMA^+$ bands was observed in all species with $2n = 38$ ('*C. acicularis*–*C. madida*' clade) and in *C. pumila*, belonging to the '*C. pumila*' clade. Species with $2n = 36$ had fewer $CMA^+$ and $DAPI^+$ bands than species with $2n = 38$. There were 2–3 $CMA^+$ blocks and 4–16 $DAPI^+$ bands in species with $2n = 36$ (Fig. 3), whereas the $2n = 38$ species (Fig. 4) exhibited 3–8 $CMA^+$ bands and 14–20 $DAPI^+$ bands (up to 32 bands in the tetraploid individuals; Fig. 4B). Among $2n = 36$ species there were always $DAPI^+$ bands present in at least one meta- or sub-metacentric pair. On the other hand,

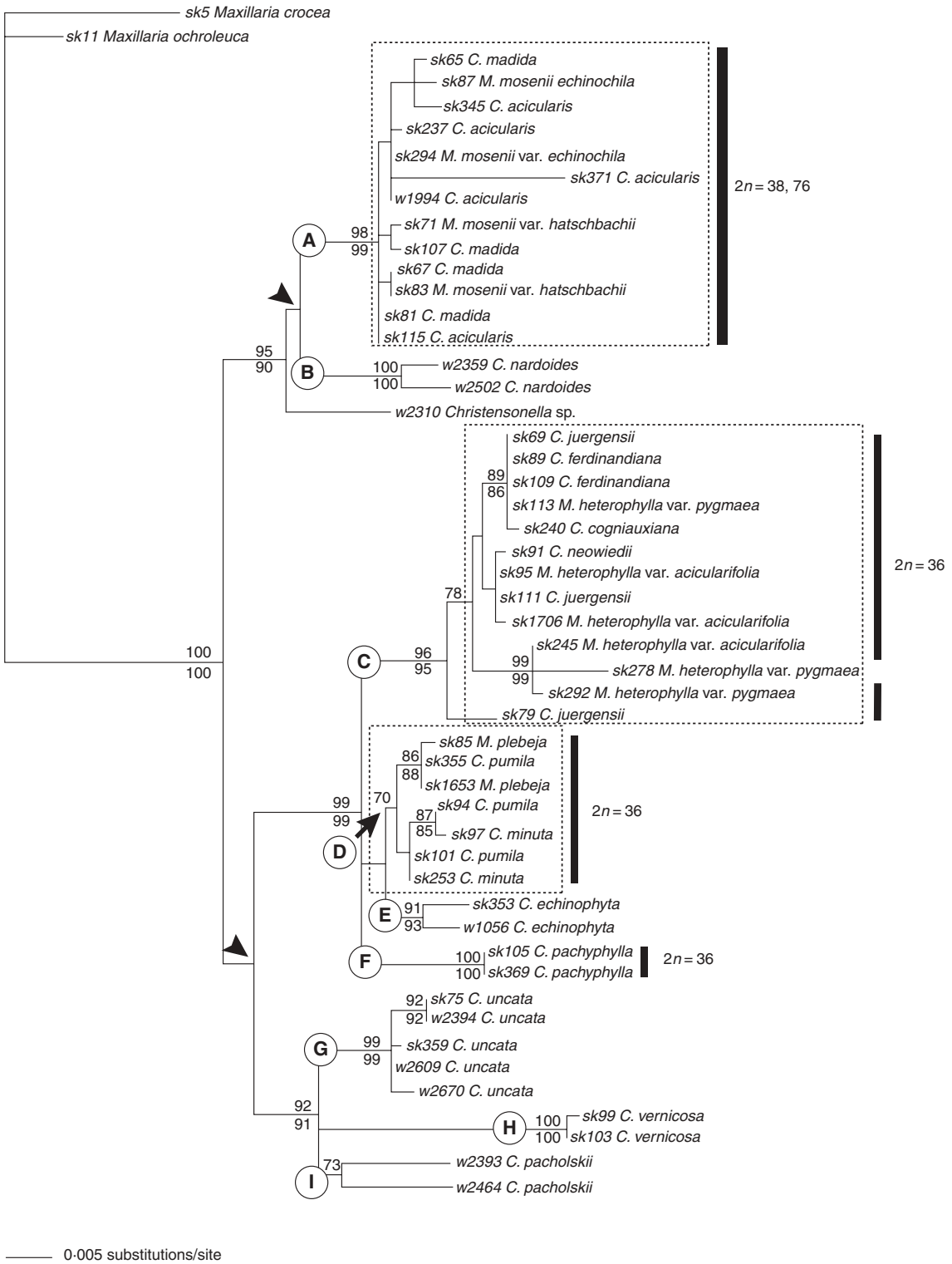
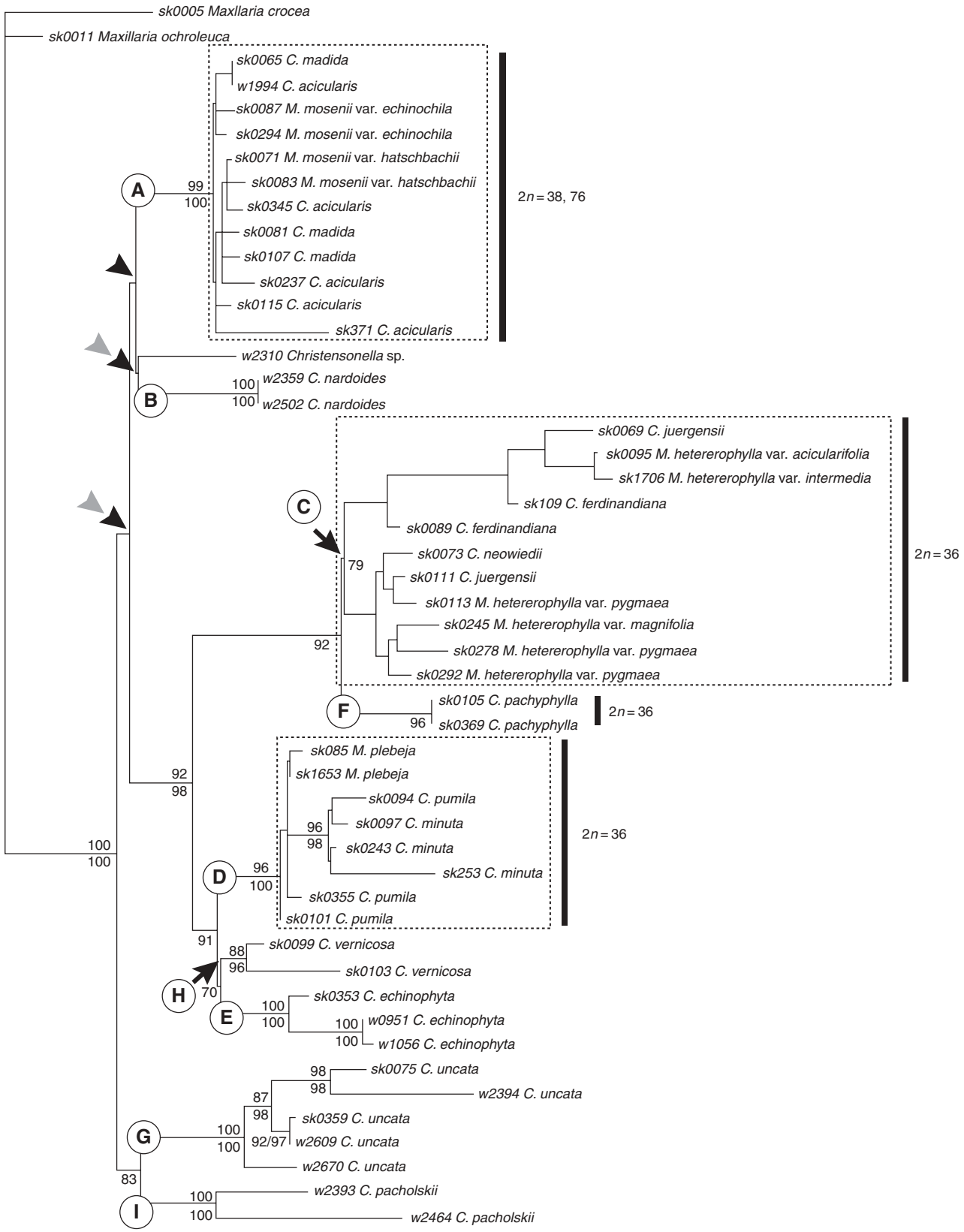



FIG. 1. Maximum likelihood phylogenies for ITS nrDNA data. Maximum likelihood/maximum parsimony bootstrap support values above 70 % are indicated above/below branches. Nodes not supported in the strict-consensus maximum-parsimony tree are indicated by arrows. In addition to *Christensonella* sp., clades recognized as species in this study are indicated by the letters A–I: (A) *Christensonella acicularis* (dotted rectangle), (B) *C. nardoides*, (C) *C. ferdinandiana* and *C. neowiedii* (dotted rectangle), (D) *C. pumila* (dotted rectangle), (E) *C. echinophyta*, (F) *C. pachyphylla*, (G) *C. uncata*, (H) *C. vernicosa*, (I) *C. pacholskii*. Chromosome numbers sampled for species regonized here are indicated by dark vertical bars.


sk0005 Maxllaria crocea
sk0011 Maxillaria ochroleuca
sk0065 C. madida
w1994 C. acicularis
sk0087 M. mosenii var. echinochila
sk0294 M. mosenii var. echinochila
sk0071 M. mosenii var. hatschbachii
sk0083 M. mosenii var. hatschbachii
sk0345 C. acicularis
sk0081 C. madida
sk0107 C. madida
sk0237 C. acicularis
sk0115 C. acicularis
sk371 C. acicularis
A
99
100
2n = 38, 76
w2310 Christensonella sp.
w2359 C. nardoides
w2502 C. nardoides
B
100
100
sk0069 C. juergensii
sk0095 M. hetererophylla var. acicularifolia
sk1706 M. hetererophylla var. intermedia
sk109 C. ferdinandiana
sk0089 C. ferdinandiana
C
79
sk0073 C. neowiedii
sk0111 C. juergensii
sk0113 M. hetererophylla var. pygmaea
sk0245 M. hetererophylla var. magnifolia
sk0278 M. hetererophylla var. pygmaea
sk0292 M. hetererophylla var. pygmaea
2n = 36
92
F
sk0105 C. pachyphylla
sk0369 C. pachyphylla
96
2n = 36
sk085 M. plebeja
sk1653 M. plebeja
sk0094 C. pumila
sk0097 C. minuta
sk0243 C. minuta
sk253 C. minuta
sk0355 C. pumila
sk0101 C. pumila
96
98
D
96
100
2n = 36
92
98
100
100
91
sk0099 C. vernicosa
88
96
sk0103 C. vernicosa
H
70
sk0353 C. echinophyta
E
100
100
100
100
w0951 C. echinophyta
w1056 C. echinophyta
98
98
sk0075 C. uncata
w2394 C. uncata
87
98
sk0359 C. uncata
G
100
100
92/97
w2609 C. uncata
w2670 C. uncata
83
w2393 C. pacholskii
I
100
100
w2464 C. pacholskii
— 0·001 substitutions/site

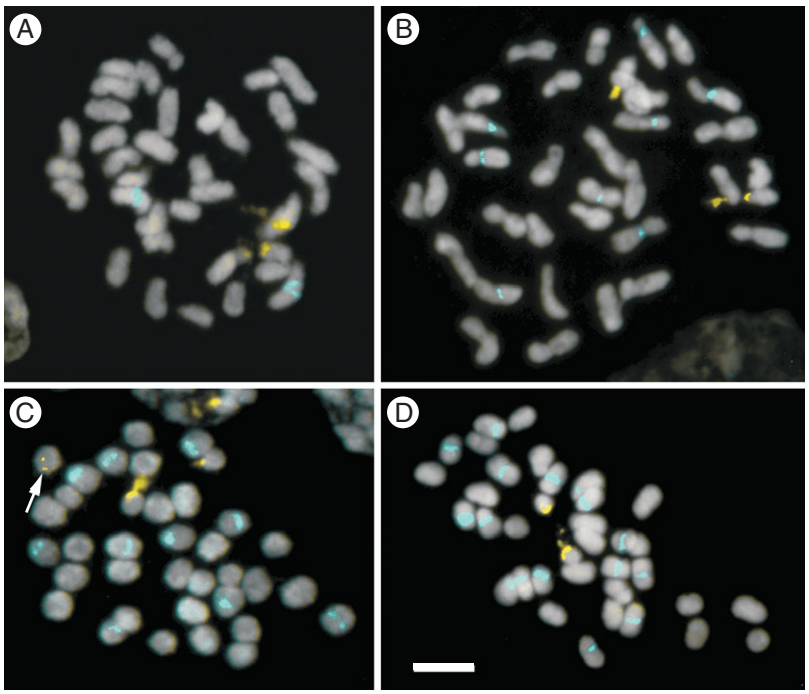



FIG. 3. Metaphase cells showing $CMA^+$ (yellow) and $DAPI^+$ (blue) banding patterns of (A) *Christensonella ferdinandiana*, (B) *C. pachyphylla*, (C) *C. pumila*, and (D) *Maxillaria heterophylla* var. *pygmaea*. Note a chromosome pair with two proximal $DAPI^+$ bands in (A). Arrow in (C) indicates a very small $CMA^+$ band. The euchromatin is grey due to the overlay of colours of both fluorochromes. Scale bar in (D) = 5 μm.

species with $2n = 38$ displayed no meta- or sub-metacentric chromosome with bands and showed both heterochromatin types, $CMA^+$ and $DAPI^+$, in some chromosome pairs (arrowheads in Fig. 4C). The species *C. ferdinandiana*, with $2n = 36$, was remarkable for bearing only one chromosome pair with duplicated $DAPI^+$ bands, presumably each one at one side of the centromere (Fig. 3A).

## DISCUSSION

### *Nuclear vs. plastid incongruence*

Incongruence among different data partitions comprises a rather complex subject in phylogenetic systematics that has received increasing attention over the last decades (Bull *et al.*, 1993; Huelsenbeck *et al.*, 1996; Cunningham, 1997; Reed and Sperling, 1999). Several studies have demonstrated that incongruence may be caused by distinct categories of bias, namely random and systematic errors and independent evolutionary histories of partitions (Swofford *et al.*, 1996; Reed and Sperling, 1999). In this study, we attempted to reduce random error by broad sampling of taxa as well as different genome regions. Sampling efforts were mainly centred on highly polymorphic species groups (the '*C. pumila*', '*C. ferdinandiana*–*C. neowiedii*' and '*C. acicularis*–*C. madida*' clades) and bootstrap analyses were performed to assess confidence in the results obtained. Despite the sampling efforts, absence of sufficient phylogenetic signal is likely to be the problem in deep levels of the recovered trees. While the ITS trees (MP and ML) suggest the (*C. pacholskii* + *C. vernicosa* + *C. uncata*) clade to be sister to the (*C. pachyphylla* + *C. echinophyta* + '*C. pumila*' + '*C. juergensii*–*C. ferdinandiana*') clade, the ML plastid topology indicates the latter (including *C. vernicosa*) as sister to the ('*C. acicularis*–*C. madida*' + *C. nardoides* + *Christensonella* sp.) clade. None of these alternatives, however, had bootstrap values greater than 50 % (Figs 1 and 2), suggesting absence of sufficient phylogenetic signal. Combined analyses (results not shown), considering both MP and ML criteria, support phylogenetic patterns indicated by the nuclear topology, but also with no bootstrap support. Soft incongruence also seems to be the reason for low resolution within and between terminal clades.

The results also suggest the occurrence of strong genealogical discordance concerning the position of *C. vernicosa*, since alternative placements of this species in nuclear and plastid trees under all search criteria received high bootstrap values (Figs 1 and 2). Four additional MP analyses were

FIG. 2. Maximum likelihood phylogenies for plastid data (atpB–rbcL spacer, trnL-F, matK regions). Maximum likelihood/maximum parsimony bootstrap support values above 70 % are indicated above/below branches. Nodes not supported in the strict consensus maximum parsimony are indicated by arrows (black arrows, unweighted analysis; grey arrows, weighted analysis). In addition to *Christensonella* sp., clades recognized as species in this study are indicated by the letters A–I: (A) *Christensonella acicularis* (dotted rectangle), (B) *C. nardoides*, (C) *C. ferdinandiana* and *C. neowiedii* (dotted rectangle), (D) *C. pumila* (dotted rectangle), (E) *C. echinophyta*, (F) *C. pachyphylla*, (G) *C. uncata*, (H) *C. vernicosa*, (I) *C. pacholskii*. Chromosome numbers sampled for species regonized here are indicated by dark vertical bars.

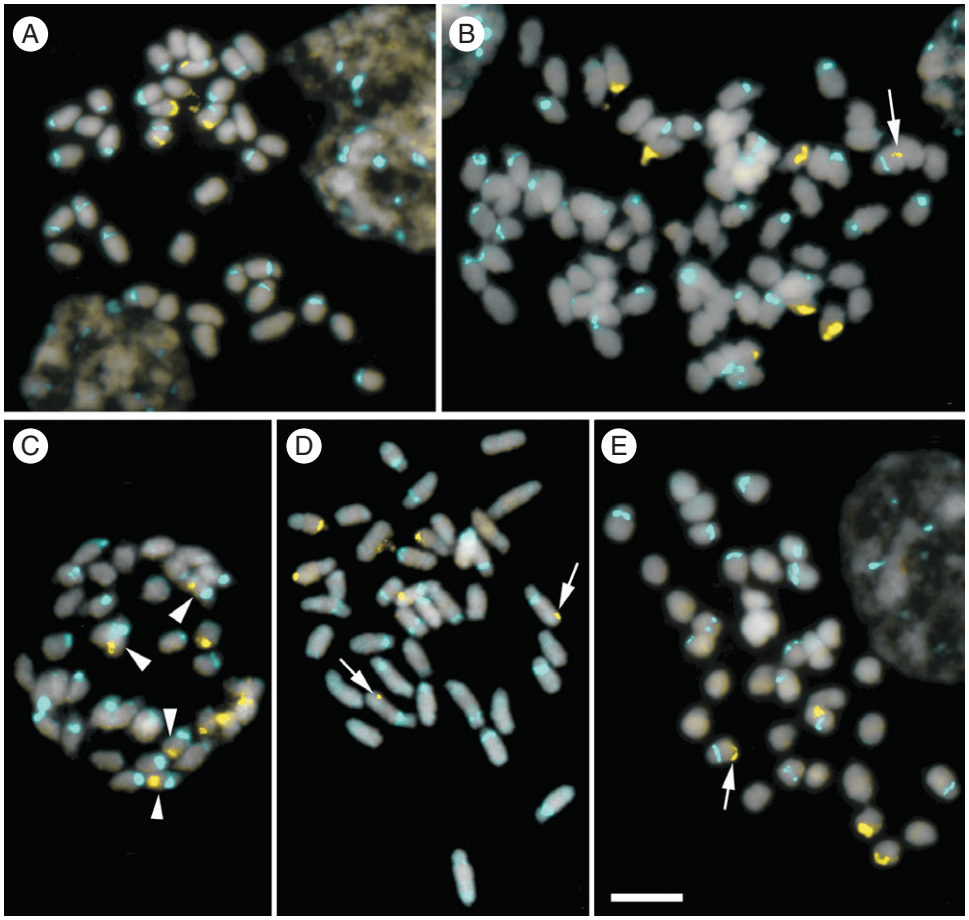



FIG. 4. Metaphase cells showing $CMA^+$ (yellow) and $DAPI^+$ (blue) banding patterns of (A) *Christensonella acicularis*, (B–C) *Maxillaria mosenii* var. *echinochila*, (D) *Christensonella madida* var. *monophylla*, and (E) *C. madida*. Arrows in (B, D, E) indicate very small $CMA^+$ bands. Arrowheads in (C) indicate the chromosomes with both $DAPI^+$ and $CMA^+$ bands. The euchromatin is grey due to the overlay of colours of both fluorochromes. Scale bar in (E) = 5 μm.

performed excluding, one at a time, outgroups as well as all samples of *C. vernicosa*, *C. echinophyta* and *C. uncata* plus *C. pacholskii* to check for possible occurrence of long-branch attraction artefacts (Siddall and Whiting, 1999), but the trees obtained did not result in any distinct topologies (results not shown). Both combined analyses (MP and ML criteria) supported the position of *C. vernicosa* as sister to (*C. pachyphylla* + *C. echinophyta* + '*C. pumila*' clade + '*C. ferdinandiana*–*C. neowiedii*' clade) with bootstrap values of 90 % and 51 %, respectively (tree not shown).

Hybridization and introgression represent potential causes of incongruence among phylogenetic trees (Mansion *et al.*, 2005; Buckley *et al.*, 2006; but see Wolfe and Randle, 2004 for additional causes of incongruence). The occurrence of recent and rapid divergence of species, sympatric populations and generalist pollinators in this group (S. Koehler, unpubl. res.) certainly reinforces the likelihood of reticulation events. Presumed hybrid individuals from natural populations bearing intermediate phenotypes have been reported for *Christensonella* (Hoehne, 1953; Onishi, 1974), although such a scenario remains to be demonstrated for *C. vernicosa*. Both lineage-sorting and reticulation processes can result in similar phylogenetic patterns (Holder *et al.*, 2001). Further studies, considering comparative analyses of a large number of uni- and biparental inherited markers and detailed geographical sampling within and between species (e.g. Comes and Abbott, 2001) are necessary to assess the role of each process in the diversification of *Christensonella*.

### *Species' delimitation*

Despite of the incongruence among topologies based on chloroplast and nuclear data sets, there was consensus concerning delimitation of monophyletic species defined by molecular data and morphological/cytological characters within *Christensonella*. Table 6 gives the current species' delimitation used to identify species for this study and the species' concepts proposed here (also indicated in Fig. 1), with diagnostic morphological characters indicated. Chromosome numbers are indicated in Figs 1 and 2. All further nomenclatural rearrangements suggested will be presented in a forthcoming taxonomic revision of the genus (S. Koehler, currently in preparation).

The molecular data presented here support the current delimitations of the species (Figs 1 and 2, Table 6): *C. nardoides*

TABLE 6. *Species delimitation as suggested in this study, with previously recognized taxa, diagnostic morphological characters and geographic distribution indicated*

| Species recognized in this study | Additional taxa recognized by previous studies, proposed here as synonyms | Diagnostic morphological characters and geographic distribution |
|---|---|---|
| *Christensonella acicularis* (Herb. ex Lindl.) Szlach., Mytnik, Górniak & Śmiszek | *C. madida* (Lindl.) Szlach., Mytnik, Górniak & Śmiszek complex; *Maxillaria mosenii* var. *echinochila* Hoehne; *Maxillaria mosenii* var. *hatschbachii* Hoehne | Plants up to 30 cm tall with uni- or bifoliate pseudobulbs, leaves variable, reddish-brown flowers with pedicels always shorter than the adjacent pseudobulb; south-eastern Brazil |
| *Christensonella echinophyta* (Barb. Rodr.) Szlach., Mytnik, Górniak & Śmiszek | – | Plants up to 5 cm tall with bifoliate pseudobulbs, needle-like leaves, white-pinkish flowers with elongate segments and pedicels always longer than the adjacent pseudobulb; south-eastern Brazil |
| *Christensonella ferdinandiana* (Barb. Rodr.) Szlach., Mytnik, Górniak & Śmiszek | – | Plants 5–10 cm tall with unifoliate and flat pseudobulbs; yellow flowers with pedicels always shorter than the adjacent pseudobulb; south-eastern Brazil |
| *Christensonella nardoides* (Kraenzl.) Szlach., Mytnik, Górniak & Śmiszek | – | Plants up to 5 cm tall; 3–4 needle-like leaves on each pseudobulb; brownish-to-red-purplish flowers with a inconspicous stipe; Bolivia, Colombia, Ecuador and Peru |
| *Christensonella pachyphylla* (Schltr. ex Hoehne) Szlach., Mytnik, Górniak & Śmiszek | – | Plants up to 25 cm tall with unifoliate cylindrical pseudobulbs; thick, lanceolate leaves; pale yellow flowers with pedicels shorter than the adjacent pseudobulbs; south-eastern Brazil |
| *Christensonella pumila* (Hook.) Szlach., Mytnik, Górniak & Śmiszek | *Christensonella minuta* (Cogn.) Szlach., Mytnik, Górniak & Śmiszek; *Maxillaria plebeja* Rchb.f | Plants up to 5 cm tall with unifoliate pseudobulbs; coriaceous-to-fleshy leaves; small yellowish-red flowers with pedicels always shorter than the adjacent pseudobulb; mostly found in south-eastern Brazil |
| *Christensonella uncata* (Lindl.) Szlach., Mytnik, Górniak & Śmiszek | – | Plants caespitose with unifoliate pseudobulbs; thick leaves; white-lavender flowers with elongate segments and stipe 2–3 mm long; central and northern South America |
| *Christensonella vernicosa* (Barb. Rodr.) Szlach., Mytnik, Górniak & Śmiszek | – | Plants up to 5 cm tall with bifoliate pseudobulbs; needle-like leaves; yellow flowers with ovate-oblanceolate segments and pedicels always longer than the adjacent pseudobulb; south-eastern Brazil |
| *Christensonella neowiedii* (Rchb.f.) S. Koehler | *Christensonella cogniauxiana* (Hoehne) Szlach., Mytnik, Górniak & Śmiszek; *Christensonella juergensii* (Schltr.) Szlach., Mytnik, Górniak & Śmiszek; *Maxillaria heterophylla* var. *acicularifolia* Hoehne; *Maxillaria heterophylla* var. *magnifolia* Hoehne; *Maxillaria heterophylla* var. *pygmaea* Hoehne | Plants up to 10 cm tall with bifoliate, cylindrical pseudobulbs; generally reddish-to-dark-purple flowers with pedicles always longer than the adjacent pseudobulb; south-eastern Brazil |
| *Christensonella pacholskii* (Schltr.) S. Koehler | – | Plants up to 5 cm tall with unifoliate pseudobulbs; leaves membranaceous and linear; flowers red-brownish with oblong segments and stipe 2–3 mm long; Ecuador |

(clade B), *C. echinophyta* (clade E), *C. pachyphylla* (clade F), *C. uncata* (clade G), *C. vernicosa* (clade H) and *C. pacholskii* (clade I), which are also easily characterized by sets of diagnostic morphological characters (Table 6). Within species from south-eastern Brazil, *C. echinophyta* (clade E, Table 6) and *C. vernicosa* (clade H, Table 6) comprise plants up to 5 cm tall with bifoliate pseudobulbs bearing needle-like leaves and flowers with pedicels always longer than the adjacent pseudobulb. *Christensonella echinophyta* has white-pinkish flowers with elongate segments, while *C. vernicosa* has yellow flowers with ovate-oblanceolate segments (Barbosa Rodrigues, 1996). Another currently recognized species from south-eastern Brazil corroborated by our results is *C. pachyphylla* (clade F, Table 6), a species growing up to 25 cm tall. It is distinguished by its unifoliate pseudobulbs bearing a thick, lanceolate leaf and pale-yellow flowers with pedicels shorter than the adjacent pseudobulbs.

The results indicate that the Amazonian species included in the genus *Christensonella* belong to two distinct subclades (Figs 1 and 2). One subclade consists of *C. nardoides* (clade B) and *Christensonella* sp. (*Whitten 2310*, an undescribed species from Peru; photograph in Butzin and Senghas, 1996, as *Maxillaria paulistana* Hoehne). *Christensonella nardoides* is easily recognized by its 3–4 needle-like leaves on each pseudobulb and brownish to red-purplish flowers with a short viscidium (Bennett and Christenson, 1993). *Christensonella* sp. is morphologically very similar to some populations of *C. madida* (= *M. madida* var. *monophylla* Cogn.), being a large plant with big, reddish flowers and unifoliate pseudobulbs.

The other north-western South American clade indicated by our results includes *C. uncata* (clade G, Table 6) and *C. pacholskii* (clade I, Table 6), which are morphologically very similar, bearing unifoliate pseudobulbs and elongated flowers with an extremely long stipe (up to 25 mm). They are easily distinguished by the colour of flowers and leaf morphology. *Christensonella pacholskii* can be recognized by its membranaceous, linear leaves and red-brownish

flowers (Christenson, 2003), whereas the widespread, vegetatively highly polymorphic *C. uncata* is easily identified by its fleshy-to-coriaceous leaves and white-to-lavender flowers (Atwood and Mora de Retana, 1999). Variation within *C. uncata* deserves further attention, and possibly two or more taxa are currently embedded in the current *C. uncata* species concept, as was suggested by Atwood and Mora de Retana (1999).

Three clades also emerged as well-supported groups of species. These are the '*C. acicularis*–*C. madida*' clade (clade A, Table 6), the '*C. ferdinandiana*–*C. neowiedii*' clade (clade C) and the '*C. pumila*' clade (clade D) (Figs 1 and 2). They all include species from south-eastern Brazil that have been shown to be very difficult to distinguish, since they are defined by continuously variable morphological characters. The '*C. pumila*' clade (clade D, Table 6) currently comprises at least three species, as demonstrated by our analyses (*C. pumila*, *M. plebeja* and *C. minuta*; Figs 1 and 2). Hoehne (1953) distinguished them based on the size and shape of leaves and on the shape and colour of flowers, but overlapping of morphological diagnostic characters is evident as soon as one attempts to identify specimens within this group. In addition, such taxa are neither geographically nor ecologically isolated, being restricted to the humid and seasonally dry forests of south-eastern Brazil. Thus, morphological variation is not reflected in geographic distribution or habitat variation, reinforcing the recognition of a single, polymorphic species for this group, namely *C. pumila*. Despite being highly polymorphic, *C. pumila* can be easily distinguished from other species of *Christensonella* by its pseudobulbs bearing one coriaceous-to-fleshy leaf and small yellowish-red flowers with pedicels always shorter than the adjacent pseudobulb.

Current species' limits within the '*C. acicularis*–*C. madida*' clade (clade A, Figs 1 and 2, Table 6) are also blurred by continuous variation of leaf shape and flower size. Plants within this clade can be distinguished from others within *Christensonella* as larger plants up to 30 cm tall, generally with reddish-brown flowers with pedicels always shorter than the adjacent pseudobulb, chromosome numbers of $2n = 38$, 76 with generally more $CMA^+$ and $DAPI^+$ (Table 3, Fig. 3). Most flowers produce a strong, fruity, watermelon-like fragrance that is very distinctive. However, in contrast to the '*C. pumila*' clade, morphological variation within '*C. acicularis*–*C. madida*' is restricted geographically and ecologically. *Christensonella acicularis* has traditionally been described as a more delicate species with bifoliate pseudobulbs, needle-like leaves and smaller flowers, and is restricted to forested habitats of south-eastern Brazil, while *C. madida* and *M. mosenii* correspond to more robust plants with larger pseudobulbs and flowers, with 1–2 leaves varying from linear-lanceolate to cylindrical. Tetraploidy was observed in two accessions of *M. mosenii* var. *echinochila* ($2n = 76$), an ecomorphotype restricted to rock outcrop formations of south-eastern Brazil. Habitat differentiation could have contributed to the initial establishment of polyploids, as autotetraploidization may be caused by high rates of formation of unreduced gametes, induced by harsh environments (Ramsey and Schemske, 1998; Soltis *et al.*, 2003). This result suggests polyploidization may have had an important role in the diversification of this group, as already demonstrated for other orchid species (Del Prete *et al.*, 1991; D'Emerico *et al.*, 2002).

Species' boundaries among *C. madida*, *M. mosenii* and *C. acicularis* have never been questioned, despite the fact there are at least six names currently available for this complex. The sequence data presented here are not informative enough to allow elucidation of the patterns of diversity within this clade. CMA/DAPI banding patterns, in general, support ecological and geographical subdivisions within this group (Table 3, Fig. 3). Further studies, utilizing more informative molecular markers at population levels and more detailed morphological and cytogenetic data, are necessary before any taxonomic change is proposed.

Another highly polymorphic well-supported clade in our analyses is the '*C. ferdinandiana*–*C. neowiedii*' clade (clade C, Figs 1 and 2, Table 6). Contrary to the other two species' complexes discussed above, there are no diagnostic morphological characters for this one. This clade comprises two morphologically very distinct groups, the currently accepted species *C. ferdinandiana* and the *C. cogniauxiana*–*C. juergensii*–*M. heterophylla*–*C. neowiedii* species' complex (or the '*C. neowiedii*' complex). The DNA sequence data do not support these two groups as distinct clades. However, *C. ferdinandiana* is morphologically very distinct from other taxa in this clade, being easily characterized by its unifoliate and flat pseudobulbs, unique in the '*C. madida*' complex, and by its yellow flowers with pedicels always shorter than the adjacent pseudobulb. Another distinctive feature of this species is the presence of a single large metacentric chromosome pair with duplicated proximal $DAPI^+$ bands (Fig. 3A). Species of the '*C. neowiedii*' complex can be distinguished from *C. ferdinandiana* by its bifoliate, cylindrical pseudobulbs and generally reddish-to-dark-purple flowers, with pedicles always longer than the adjacent pseudobulb. In contrast to *C. ferdinandiana*, species' delimitation within the '*C. neowiedii*' complex is extremely unclear and vague, since diagnostic characters once again vary continuously among taxa. Such intricate morphological variation is well illustrated by the fact that it is possible to observe in the same locality, such as Campos de Jordão (São Paulo State, Brazil), individuals with flat, lanceolate leaves and reddish, smaller flowers blooming together with needle-leaved specimens with larger, dark-purple flowers, with intermediate morphotypes growing between them.

One of the problems with the phylogenetic species' concept is dealing with recent diverged lineages, since they usually are not reciprocally monophyletic (Coyne and Orr, 2004). Clearly the molecular markers used in this study did not present enough variation to distinguish between *C. ferdinandiana* and the '*C. neowiedii*' group, despite the fact that morphology and cytogenetic data do separate them. Considering our present lack of knowledge of phylogenetic patterns within this group, we suggest a conservative approach for species' delimitation for the '*C. ferdinandiana*–*C. neowiedii*' clade based on morphological characters. Although our results do not support any of these entities as monophyletic, morphological characters can be used, at least in a first instance, to

distinguish *C. ferdinandiana* and a broad '*C. neowiedii*', as described above.

*Cytogenetic patterns of diversification*

Cytogenetic data gathered for *Christensonella* show two general patterns. The species *C. ferdinandiana*, *C. pachyphylla*, *C. pumila* and *M. heterophylla* are characterized by $2n = 36$, few $CMA^+$ bands and occurrence of sub-meta- and metacentric chromosomes, while *C. acicularis*, *C. madida* and *M. mosenii* have karyotypes with $2n = 38$, more $CMA^+$ bands and an apparent lack of sub-meta- and metacentric chromosomes. While $2n = 38$, 40 has been shown to occur in most of the taxa sampled for the core subtribe Maxillariinae (*sensu* Whitten *et al.*, 2007), the occurrence of chromosome numbers of $2n = 36$ is much more restricted in this group. Only 12 out of the 68 taxa sampled for chromosome numbers in the core Maxillariinae have $2n = 36$ or less: $2n = 28$ for *Cryptocentrum standleyi*; $2n = 30$ for *C. lehmanii*; $2n = 32$ for *M. arachnitiflora*, $2n = 34$ for *M. fulgens*, *M. hedwigae*, *M. notylioglossa* and *M. rufescens*; and $2n = 36$ for *M. barbosae*, *M. bicallosa*, *M. desvauxiana*, *M. microdendron* and *M.* cf. *luteoalba* (Blumenschein and Paker, 1963; Carnevali 1991; Carnevali Fernandez-Concha, 1996; Brandham, 1999; Felix and Guerra, 2000; Whitten *et al.*, 2007).

Disploidy has already been indicated as a mechanism of karyotype evolution in other groups of orchids, as in the section *Fimbriatae* of *Lycaste* (Ryan *et al.*, 2000) and in the genera *Cephalanthera* (Schwarzacher and Schweizer, 1982) and *Paphiopedilum* (Cox *et al.*, 1998). The probable mechanism involved in the dysploid differentiation of chromosome numbers in *Christensonella* is centric fusion or fission, as suggested by the presence of a single, large metacentric chromosome pair with duplicated proximal $DAPI^+$ bands in *C. ferdinandiana*. The fact that acro- or telocentric chromosomes with proximal $DAPI^+$ bands have been observed in the species studied with $2n = 38$ and that all the sampled species with $2n = 36$ had at least one sub-meta- or metacentric chromosome pair with a proximal $DAPI^+$ band, not found in any sampled species with $2n = 38$, suggests the occurrence of fusion/fission changes.

Felix and Guerra (2000) had earlier suggested that the probable chromosome base number for *Maxillaria* is $x_2 = 20$, which places the numbers $2n = 36$, 38 as evolving from a sequence of descendent disploidy events from $2n = 40$. In this scenario, the ancestral condition for chromosome numbers in *Christensonella* would be $2n = 38$ and the '*C. acicularis*–*C. madida*' clade would have conserved the plesiomorphic state of chromosome number in the genus. Moreover, the common ancestor of species with $2n = 36$ would have experienced further descendent disploidy due to centric fusion resulting in at least one sub-meta- or metacentric chromosome pair. Preliminary data on chromosome counts of *C. uncata* confirmed the occurrence of $2n = 36$ for this species (J. S. Cabral, unpubl. res.). This information supports the phylogenetic tree based on nuclear (and combined) sequence data, as it places *M. uncata* as the sister clade of the other species with $2n = 36$ (Fig. 1).

Nevertheless, little is still known about patterns of karyotypic evolution in the Maxillariinae subtribe. Only 68 taxa of the 354 species recognized for the core subtribe Maxillariinae have been sampled for chromosome numbers (see review in Whitten *et al.*, 2007). The evolution of chromosome numbers in *Christensonella* could not be fully assessed by the phylogenetic and karyotypic data available. The sister group of *Christensonella* is still unclear (Whitten *et al.*, 2007) and plastid and nuclear topologies presented here indicate conflicting patterns of diversification within this group. Further cytogenetic studies, considering additional samples within Maxillariinae as well as complementary phylogenetic data, are necessary for an accurate inference of the evolution of cytogenetic patterns in *Christensonella.*

## CONCLUSIONS

DNA sequence data and cytological analyses were used to investigate species' boundaries within the neotropical *Christensonella*. Six currently accepted species were recovered by the phylogenetic analyses presented here (*C. echinophyta*, *C. nardoides*, *C. pachyphylla*, *C. pacholskii*, *C. vernicosa* and *C. uncata*). Our results also support the recognition of the '*C. pumila*' clade as a single, polymorphic species based on diagnostic morphological characters as well as by an overlapping geographic distribution of current species recognized within this clade. Two additional clades, the '*C. acicularis*–*C. madida*' clade and the '*C. ferdinandiana*–*C. neowiedii*' clade, demand further investigation since patterns of diversification remained obscured within both groups. For now, we propose the recognition of a broadly defined *C. acicularis* for the '*C. acicularis*–*C. madida*' clade and two morphological complexes within the '*C. ferdinandiana*–*C. neowiedii*' clade (namely *C. ferdinandiana* and *C. neowiedii s.l.*) based on diagnostic morphological features described above. Complementary studies considering more populations as well as data from different molecular markers are already being developed in order to better understand diversification patterns within these clades. Patterns of heterochromatin distribution as well as karyotypic data certainly deserve greater attention as a valuable complementary source of information to understand evolutionary patterns within Maxillariinae, as well as to assist in the revision of species/generic boundaries within this subtribe. It is clear that speciation within Orchidaceae is a complex issue that must be explored in greater detail. The question of how such a vast range of morphological diversity was shaped and what is the role of ecology behind the formation of new species remains a fascinating subject to be further explored in this group of plants.

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## LITERATURE CITED

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

**Barbosa Rodrigues J. 1996.** *Maxillaria echinophyta* Barb. Rodr. In: Sprunger S., ed. *Iconographie des Orchidées du Brésil. Vol. 1: The Illustrations.* Basle: Friedrich Reinhardt Verlag.

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

**Blanco MA, Carnevali G, Whitten WM, Singer RB, Koehler S, Williams NH, *et al*. 2007.** Generic realignments in Maxillariinae (Orchidaceae). *Lankesteriana* **7**: 515–537.

**Blumenschein A, Paker IU. 1963.** Número de cromossomas nas Maxillariinae (Orchidaceae). *Ciência e Cultura* **15**: 255.

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

**Brasileiro-Vidal AC, dos Santos-Serejo JA, Soares Filho W dos S, Guerra M. 2007.** A simple chromosomal marker can reliably distinguish *Poncirus* from *Citrus* species. *Genetica* **129**: 273–279.

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

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

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

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

**Carnevali G. 1991.** *The cytology and the pollinaria in the Maxillariinae (Orchidaceae).* M.S. thesis, University of Missouri, St. Louis, Missouri, USA.

**Carnevali Fernandez-Concha G. 1996.** *Systematics, phylogeny and twig epiphytism in* Cryptocentrum *(Orchidaceae).* Ph.D. thesis, University of Missouri, St. Louis, Missouri, USA.

**Carpenter JM. 1994.** Successive weighting, reliability and evidence. *Cladistics* **10**: 215–220.

**Christenson EA. 2002.** Vue d'ensemble du genere *Maxillaria*. *Richardiana* **2**: 41–65.

**Christenson EA. 2003.** Two *Maxillarias*, one old and one new. *Orchid Review* **111**: 287–289.

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

**Comes HP, Abbott RJ. 2001.** Molecular phylogeography, reticulation, and lineage sorting in Mediterranean *Senecio* sect. *Senecio* (Asteraceae). *Evolution* **55**: 1943–1962.

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

**Coyne JA, Orr HA. 2004.** *Speciation*. Sunderland, MA: Sinauer Associates, 459–470.

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

**D'Emerico S, Cozzolino S, Pellegrino G, Pignone D, Scrugli A. 2002.** Karyotype structure, supernumerary chromosomes and heterochromatin distribution suggest a pathway of karyotype evolution in *Dactylorhiza* (Orchidaceae). *Botanical Journal of the Linnean Society* **138**: 85–91.

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

**Dobigny G, Ducroz J-F, Robinson TJ, Volobouev V. 2004.** Cytogenetics and cladistics. *Systematic Biology* **53**: 470–484.

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

**Farris JS. 1969.** A successive approximation approach to character weighting. *Systematic Zoology* **18**: 374–385.

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

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

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

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

**Goldman DH, Freudenstein JV, Kores PJ, Molvray M, Jarrell DC, Whitten WM, *et al*. 2001.** Phylogenetics of Arethuseae (Orchidaceae) based on plastid *matK* and *rbcL* sequences. *Systematic Botany* **26**: 670–695.

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

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

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

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

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

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

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

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

**Johnson LA, Soltis DE. 1994.** *MatK* DNA sequences and phylogenetic reconstruction in Saxifragaceae *s.s. Systematic Botany* **19**: 143–156.

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

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

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

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

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

**Rambaut A. 2002.** *Se-Al Sequence Alignment Program, version 2·0a11*. Department of Zoology, University of Oxford, Oxford, UK. Available at http://evolve.zoo.ox.ac.uk/.

**Ramsey J, Schemske DW. 1998.** Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review in Ecology and Systematics* **29**: 467–501.

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

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

**Schwarzacher T, Schweizer D. 1982.** Karyotype analysis and heterochromatin differentiation with Giemsa C-banding and fluorescent counterstaining in *Cephalanthera* (Orchidaceae). *Plant Systematics and Evolution* **141**: 91–113.

**Schweizer D, Ambros PF. 1994.** Chromosome banding. In: Gosden JR, ed. *Methods in molecular biology*, vol. 29. Totowa, NJ: Humana Press, 97–112.

**Siddall ME, Whiting MF. 1999.** Long-branch abstractions. *Cladistics* **15**: 9–24.

**Soltis DE, Soltis PS, Tate JA. 2003.** Advances in the study of polyploidy since plant speciation. *New Phytologist* **161**: 173–191.

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

**Swofford DL. 2000.** *PAUP*: Phylogenetics analysis using parsimony (and other methods), version 4·0b10*. Sunderland, MA: Sinauer Associates.

**Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996.** Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*, 2nd edn. Sunderland, MA: Sinauer, 407–514.

**Szlachetko DL, Mytnik-Ejsmont J, Górniak M, Śmiszek M. 2006.** Genera et species orchidalium. 15. Maxillarieae. *Polish Journal of Botany* **51**: 57–59.

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

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

**Whitten WM, Williams NH, Blanco MA, Endara L, Neubig K, Koehler S, Carnevali G, Singer R. 2007.** Molecular phylogenetics of *Maxillaria* and related genera (Orchidaceae: Cymbidieae) based upon combined molecular data sets. *America Journal of Botany* **94**: 1860–1889.

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