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Phylogenetic relationships within the subtribe Spiranthinae *s.l.* (Orchidaceae) inferred from the nuclear ITS region

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Abstract: Results of an analysis of ITS sequences for the subtribe Spiranthinae *s.l.* (Orchidaceae) are presented, and compared with results of morphological studies. To evaluate the monophyly of the subtribe, nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene were sequenced for 19 taxa of the tribe Spirantheae, with *Erythrodes* sp., *Bonatea speciosa*, *Cynorkis* sp., *Pterostylis curta* and *Chloraea flavescens* designated as an outgroup. For 8 taxa, sequences were taken from the GenBank. The results confirmed the current subtribal position of Cyclopogoninae, with members of the subtribe forming a moderately supported clade. The unexpected occurrence of *Odontorrhynchos* (Spiranthinae) within the Cyclopogoninae is discussed with reference to the results of morphological studies. Although some of the genera representing the subtribe Stenorrhynchidinae Szlach. form a weakly supported clade, the remaining taxa are variously grouped with Spiranthinae representatives, receiving moderately weak bootstrap support. The results do not provide evidence for the strict monophyly of Spiranthinae *s.l.* and Stenorrhynchidinae.

Key words: Orchidaceae, Spiranthinae, Cyclopogoninae, *Odontorrhynchos*, molecular taxonomy, ITS

1. Introduction

The generic and species composition of Spiranthinae, due to its high polymorphism, has stirred up a lot of controversy from the beginning. Most of its taxa were initially included in the genus *Spiranthes* L. C. Rich., but Schlechter (1920) divided it into 24 genera. His work was harshly criticized by other scientists, mainly from the United States (Ames 1922; Williams 1951; Schweinfurth 1958). They accused him of inconsistent usage of criteria to distinguish the taxa and, most of all, subjective choice of diagnostic features. In the 1980's, Garay (1982) and Burns-Balough (1982) independently undertook attempts to revise the Spiranthinae. Their results turned out to diverge to a large extent, so Szlachetko made further attempts to verify the classification of Spiranthinae (Szlachetko 1991a, 1991b, 1992a-e, 1993a-e, 1994a-c; Szlachetko & Tamayo 1996; Tamayo & Szlachetko 1998). He also introduced a new concept of the tribe Spirantheae (Szlachetko 1995), into which he included Prescottiinae, Cyclopogoninae, Stenorrhynchidinae and Spiranthinae.

The subtribe Cyclopogoninae Szlach., as defined by Szlachetko (1995), embraces 9 genera. The distinguishing feature of this group is the characteristic viscidium produced on the dorsal surface of the rostellum. Its lower, firm layer is built of sclerenchymatous cells, while the upper, soft layer is made of partially macerated cells. The viscidium is enclosed by wishbone-like pollinium apices, and caudiculae are lamellar. The rostellum is soft, ribbon-like, entire or apically furculate, and partly surrounds the pollinarium. However, the generic delimitations between the largest genera, i.e. *Cyclopogon*, *Pelexia* and *Sarcoglottis*, are not clear (Szlachetko *et al.* 2005). At first, Szlachetko (1995) distinguished 7 genera within Cyclopogoninae: *Pelexia* Poit. *ex* L.C. Rich. (1818), *Cyclopogon* Presl (1827), *Sarcoglottis* Presl (1827), *Stigmatosema* Garay (1982), *Cocleorchis* Szlach. (1994), *Warscaea* Szlach. (1994), and *Veyretia* Szlach. (1995). A few years later he proposed

additional genera: *Zhukowskia* Szlach., R. Gonzalez T. & Rutk. (2000), *Pachygenium* (Schltr.) Szlach., R. Gonzalez T. & Rutk. (2001), and *Potosia* (Schltr.) R. Gonzalez T. & Szlach. (2003). Currently Cyclopogoninae, as delimited by Szlachetko, embrace 10 genera. Such a group is also consistent with results of palynological studies by Balogh (1982).

The subtribe Stenorrhynchidinae Szlach., proposed by Szlachetko (1995), includes about 20 genera. The distinguishing features are: the sheath-like viscidium, produced of the outer layer of rostellum cells, and the subulate rostellum remnant. Stenorrhynchidinae have 2 distinct centres of diversity, located almost symmetrically in relation to the equator: in central Mexico, somewhat below the tropic of Cancer (Jalisco, Michoacan, Veracruz, San Luis Potosi), and in south Brazil, near the tropic of Capricorn (Espirito Santo, Rio de Janeiro and São Paulo).

The subtribe Spiranthinae Lindl. *sensu* Szlachetko 1995 embraces about 30 genera. The distinguishing features are: the viscidium produced on the adaxial layer of rostellum cells, and the deeply notched or foveolate rostellum remnant.

In contrast, Dressler (1993) proposed different subtribal and tribal concepts, with Spirantheae *sensu* Szlachetko in the subtribal rank within Cranichideae Endl. Following his scheme, Salazar *et al.* (2003) conducted a phylogenetic analysis of Cranichideae, with special focus on Spiranthinae *sensu* Dressler (1993). That study involved 42 species representing all the subtribes of Cranichideae of Dressler's (1993) concept, including taxa recognized by Szlachetko as Cyclopogoninae. Nevertheless, the study conducted by Salazar *et al.* (2003) comprised only 3 taxa of Cyclopogoninae, representing the genera *Cyclopogon*, *Pelexia* and *Sarcoglottis*. In our opinion their sampling strategy was not extensive enough to clearly evaluate the taxonomic status of the subtribe. Members of Cyclopogoninae formed a nearly monophyletic clade, disrupted by the occurrence of *Odontorrhynchos variabilis* Garay. The genus *Odontorrhynchos* Correa has been included by Szlachetko in Spiranthinae. According to extensive morphological data, those genera of Cyclopogoninae and *Odontorrhynchos* are only distantly related.

To verify the subtribal boundaries we decided to expand the sampling of the taxa representing Cyclopogoninae and to include additional taxa representing genera of subtribes Spiranthinae and Stenorrhynchidinae *sensu* Szlachetko. To evaluate the monophyly of the subtribe, we sequenced the nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2), and the 5.8S gene, which is a part of the nrDNA multigene family. Earlier analyses of ITS sequences of orchids succeeded to determine the degree of relationship on the subtribe level: Spiranthinae (Salazar *et al.* 2003), Laeliinae (Berg *et al.* 2000), Disinae (Douzery *et al.* 1999), Pogoniinae (Cameron & Chase 1999), Orchidinae (Pridgeon *et al.* 1997), Pleurothallidinae (Pridgeon *et al.* 2001); and on the generic level: *Stanhopea* (Whitten *et al.* 2000), *Lycaste* and *Anguloa* (Ryan *et al.* 2000), *Cypripedium*, *Selenipedium* and *Paphiopedilum* (Cox *et al.* 1997). Also Bateman's (1997) work shows that ITS is very helpful to determine phylogenetic relations of closely related genera and species within the Orchideae. To provide a basis for a clear distinction of Cyclopogoninae *sensu* Szlachetko, we compared the results of our phylogeneticanalysis with results of morphological studies.

2. Material and methods

2.1. Sampling of taxa

Nineteen taxa representing Spirantheae *sensu* Szlachetko were originally selected for this study, with *Erythrodes* sp., *Bonatea speciosa*, *Cynorkis* sp., *Pterostylis curta* and *Chloraea flavescens* designated as the outgroup. For 8 taxa, sequences were taken from the GenBank: *Burnsbaloghia diaphana* (AJ 539484), *Funkiella hyemalis* (AJ 539495), *Mesadenus lucayanus* (AJ 539488), *Odontorrhynchos variabilis* (AJ 539498), *Schiedeella llaveana* (AJ 539487), *Spiranthes cernua* (AJ 539495), *Stenorrhynchos aurantiacus* (AJ 539485), *Stenorrhynchos speciosum* (AJ 539505). Vouchers and GenBank accession number for new taxa are available upon request from first author.

2.2. Amplification and sequencing

Total genomic DNA was extracted from 100 mg of fresh-frozen or 20 mg of silica-dried leaves (Chase & Hillis 1991) by using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to manufacturer's protocol.

The ITS region (ITS1-5.8s-ITS2) was amplified via polymerase chain reaction (PCR) in a Biometra T1 thermal cycler. PCR was carried out in a volume of 50 µl. The PCR mixture contained: dd H_2O, 5 µl 10 × polymerase buffer with 15 mM $MgCl_2$, 1 µl of 10mM mix of each dNTP (200 µM), 10 µl 5 × Qsolution, 0.5 µl of 20 mM ITS4 and ITS5 primers (White et al. 1990), 2.5 units of Taq DNA Polymerase (Qiagen), and genomic DNA. The thermal cycling protocol of the PCR consisted of 4 min of initial denaturation at 94°C, followed by 30 cycles, each with 45 sec of denaturation at 94°C, 45 sec of annealing at 52°C and 45 sec of extension at 72°C, and ending with 5 min of extension at 72°C.

Amplified products were cleaned with a High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Mannheim, Germany), according to manufacturer's protocol.

Cycle sequencing was carried out directly on the purified product by using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, Cheshire, UK): 2 µl of sequencing buffer, 4 µl of Big Dye Terminator with 4 µl of 0.08 mM primer (3.2 pmol), 5-8 µl of amplified product, and dd H_2O in a total reaction volume of 20 µl. The PCR primers ITS4 and ITS5 (White *et al.* 1990) were used to sequence both strands of the ITS region. Cycle sequencing conditions for those strands were as follows: 20 sec of initial denaturation, followed by 25 cycles, each with 15 sec of denaturation at 94°C, 20 sec of annealing at 52°C, and 4 min of elongation at 60°C, by using a Biometra thermal cycler. Sequencing reactions were purified with an ExTerminator Kit (A&A Biotechnology, Gdynia, Poland), according to manufacturer's protocol. Pelleted samples were sequenced on an Applied Biosystems 377 automated sequencer. Forward and reverse strands were sequenced to assure accuracy in base calling.

2.3. Phylogenetic analyses

DNA sequences were aligned by 'ClustalX™' and adjusted manually. ITS sequences were analysed by using the heuristic search method of PAUP* version 4.0b10 (Swofford 2000). The optimality criterion was parsimony with tree-bisection-reconnection (TBR) branch swapping and the MULTREES option in effect, simple addition and ACCTRAN optimization. Gaps were coded as missing values. Internal support of clades was evaluated by the bootstrap (Felsenstein 1985) method with 1000 bootstrap replicates. All characters were unordered and equally weighted.

3. Results and discussion

The ITS region included a total of 760 aligned positions, of which 348 were constant, and 98 variable characters were parsimony-uninformative. There were 314 parsimony-informative characters. Figure 1 depicts a single, 50% majority rule bootstrap consensus tree, with a length of 1143 steps, CI (consistency index) of 0.55 and RI (retention index) of 0.66. Four groups can be identified, with Prescottiinae as a monophyletic sister group with high bootstrap (BP) support in relation to the rest of spiranthoids (BP 100). Cyclopogoninae (*Cyclopogon*, *Pelexia* and *Sarcoglottis*) with *Odontorrhynchos* form a well-supported clade (BP 97). *Sauroglossum* Lindl., with moderately weak support (BP 72) are a sister group to the latter (clade A). The position of *Coccineorchis* Schltr. remains unresolved. The results do not provide evidence for the strict monophyly of Spiranthinae *sensu* Szlachetko (1995) and Stenorrhynchidinae Szlach. Although some of the genera representing the latter subtribe form a weakly supported clade B (BP 76), the remaining taxa are variously grouped with Spiranthinae representatives, receiving moderately weak bootstrap support (BP 75 – clade C).

Cyclopogoninae (clade A)

This clade consists of taxa that represent Cyclopogoninae *sensu* Szlachetko 1995 (*Cyclopogon, Sarcoglottis* and *Pelexia*). Szlachetko (1995) singled out Cyclopogoninae from the subtribe Spiranthinae *sensu* Dressler (1993) on the basis of the difference in structure of the viscidium and rostellum (see above). Previously Greenwood (1982) noted that *Cyclopogon, Sarcoglottis* and *Pelexia* have the specific type of viscidium (wedge-type viscidium), which was also confirmed by Burns-Balogh & Robinson (1983). *Sauroglossum* (a sister group to the Cyclopogoninae and *Odontorrhynchos* clade) is similar to *Odontorrhynchos* and *Brachystele* with respect to the structure of the column and perianth. Is there any explanation of the close relationship of *Sauroglossum* to Cyclopogoninae? Gerardo Salazar (2001) in *Genera Orchidacearum* (2001) noted that *Sauroglossum* shares many features with *Pelexia*: a long slender column, ovate cordate anther with an apical projection, and also lip structure similar to that of *Pelexia weberbaueriana* Schltr. On the other hand, Szlachetko (1995) on the basis of the structure of the column, especially the rostellum and viscidium, placed *Sauroglossum* in Spiranthinae. Molecular data and some morphological features indicate that *Sauroglossum* and Cyclopogoninae could have had the same ancestor. However, many differences, especially the structure of the column, eliminate *Sauroglossum* from the subtribe Cyclopogoninae. The clade is well supported and constitutes a compact group, except the unusual position of *Odontorrhynchos*. That genus represents the subtribe Spiranthinae *sensu* Szlachetko (1995) or „*Brachystele* alliance" *sensu* Balogh (1982). The differences between *Odontorrhynchos* and *Pelexia* are very significant, as those genera differ in many morphological characters regarded as evolutionarily conservative. In *Odontorrhynchos* the gynostemium is short and rather massive, its column part shorter than the anther, and the column foot is rudimentary, whereas *Pelexia* is characterized by an elongate, slender gynostemium with the column part being longer than the anther, and a prominent column foot. The genera differ in the presence (*Pelexia*) or absence (*Odontorrhynchos*) of caudiculae, spur and claw of the lip. Those orchid taxa also exhibit different kinds of viscidium, which is small, elongate, produced on the dorsal surface of the rostellum apex, and glued apically (acrotonically) with pollinia in *Pelexia*, and produced on the central part of the rostellum base and glued to the basal part of pollinia (basitonically) in *Odontorrhynchos*. The rostellum remnant is tridentate and fleshy in the latter genus, and linear with foveolate apex, slender and soft in the former.

Pelexia has oblique petals and lateral sepals, which are decurrent on the column foot, whereas tepals in *Odontorrhynchos* are free and straight. Besides, the genera differ in lip morphology, especially in the hypochile structure. In *Odontorrhynchos* it is sigmoid, its margins are folded, and the basal part is saccate, but the hypochile in *Pelexia* is slightly concave near the top, and the epichile is strongly bent forward. Auricles are produced on the outer margin of the lip in the former genus, but they are produced on the inner part of the lip margin in the latter one. Also leaves differ in shape and proportions: those of *Odontorrhynchos* are oblong-lanceolate

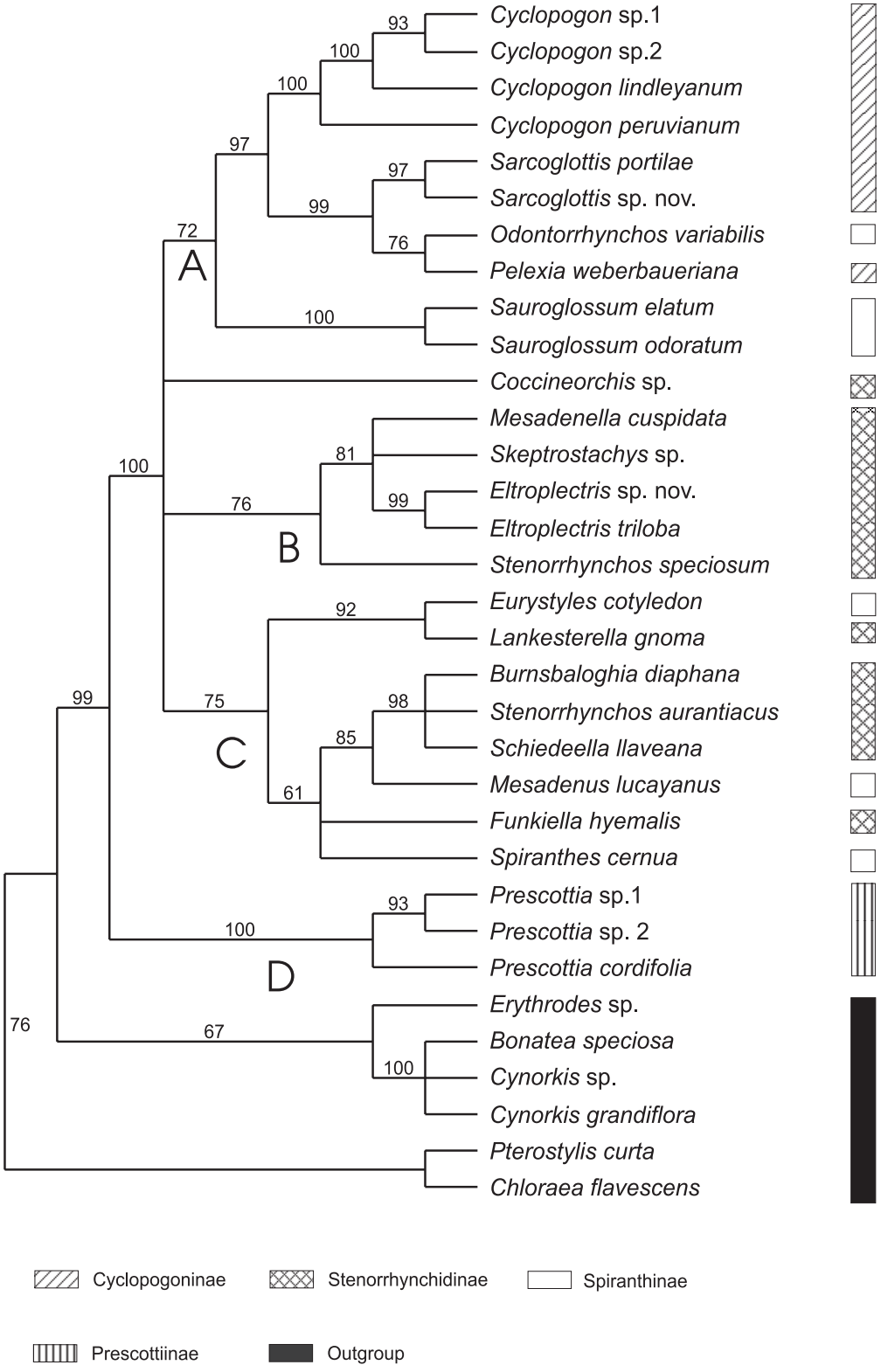

Fig. 1. Bootstrap 50% majority rule consensus tree from the parsimony analysis of ITS sequences from taxa of the tribe Spirantheae. Bootstrap percentages >50 are indicated above the branches; A, B, C, D – clades

with short and wide petioles, whereas those of *Pelexia* are oblong-ovate with long and narrow petioles. In our opinion it is not possible for both taxa to have evolved. As an explanation we suggest that plant material for the genus *Odontorrhynchos*, used in the study by Salazar *at al*. (2003), could have been determined incorrectly, leading to a continuous sampling error. It could have been a plant of the genus *Pelexia* or *Cyclopogon*, or another representative of Cyclopogoninae closely related to *Pelexia*.

Spiranthinae and Stenorrhynchidinae (clades B and C)

As mentioned earlier, our results do not support a strict monophyly of Spiranthinae (with a notched rostellum remnant) and Stenorrhynchidinae (with a subulate rostellum) *sensu* Szlachetko, which is congruent with the report by Salazar et *al*. (2003). This author suggest that the structure of the rostellum and viscidium has been determined by pressures from pollinators. According to Szlachetko *et al*. (2005) this explanation is impossible because it implies that 'the notched rostellum remnant and viscidium, formed of the apical part of the rostellum, evolved independently 5 times'. The resulting consensus tree (Fig. 1) shows some weak evidence that the subtribe Spiranthinae *sensu* Szlachetko tends to be more polyphyletic than Stenorrhynchidinae. However, we were unable to extend the sampling of taxa and verify this.

It is worth noting that many genera from both subtribes seem to be more closely related than indicated by results of morphological studies. An example is the close relationship between *Eurystyles cotyledon* Wawra and *Lankesterella longicollis* (Cogn.) Hoehne. Both taxa were classified by Szlachetko (1995) within different subtribes, Spiranthinae and Stenorrhynchidinae, respectively.

Eurystyles was described by an Austrian, Heinrich Wawra, in 1863, and considering the unusual inflorescence for the order Orchidales, it was initially classified into the order Zingiberales by mistake. Schlechter (1920) included 4 species into *Eurytyles*, which was described by him earlier as a new genus *Trachelosiphon*: *T. actinosophilum* (Barb. Rodr.) Schltr., *T. ananassocomos* Schltr., *T. cogniauxii* (Kraenzl.) Schltr., and *T. lorenzii* (Cogn.) Schltr. In the same work he also described the genus *Cladobium* (today's *Lankesterella* Ames) with 5 Brazilian species, which were formerly included into *Stenorrhynchos* Rich. *ex* Spreng.: *C. ceracifolium* (Barb.Rodr.) Schltr., *C. epiphytum* (Barb.Rodr.) Schltr., *C. gnomus* (Kraenzl.) Schltr., *C. longicolle* (Cogn.) Schltr. and C. *pilosum* (Cogn.) Schltr. In that work and in the next one, Schlechter (1920, 1926) placed *Trachelosiphon* and *Cladobium* in different groups of the tribe Spirantheae because of differences in gynostemium and viscidium structures. Since then, opinions about the degree of affinity between *Eurystyles* and *Lankesterella* have varied. Dressler (1981), and Salazar *et al*. (2003) also suggested a close relation between those taxa and consequently they postulated joining them.

Both genera assemble small epiphytic plants with fasciculate, fleshy, puberulent roots. Their leaves are basal, rosulate, with narrow petioles. Blades are ovatelanceolate to oblanceolate, acute, with ciliolate margins. The scape is erect to arcuate, delicate, and densely villose. The inflorescence of *Eurystyles* is very dense, all-sided, supported at the base by few sterile bracts, so it resembles the inflorescence of Asteraceae. The raceme of *Lankesterella* is few-flowered and lax. Flowers of *Eurystyles* are non-resupinate, which makes this genus unusual not only in comparison to *Lankesterella*, but to all Spirantheae. Beside *Eurystyles*, non-resupinate flowers are found only in *Hapalorchis trilobata* Schltr. *Pseudoeurystyles* Hoehne and *Aracamunia* Carnevali & I.Ramirez.

In *Lankesterella* lateral sepals are connate and form a chin-like or conical-cylindrical spur, while the lip is sessile and in *Eurystyles* the lip is clawed. The genera differ in lip structure. The claw in *Eurystyles* is often wide and concave, with basal auricles, often terminated by thick appendages. There are also many differences between the two genera in gynostemium structure. It is elongate and slender in *Eurystyles* and relatively short in *Lankesterella*. The column foot is prominent and rather massive in the latter genus, and short, incurved and adnate to the ovary apex in the former. Pollinia in *Eurystyles* are unequal in size and shape, and free from caudiculae, whereas in *Lankesterella*, caudiculae are present in most species. The stigma in *Eurystyles* is 3-lobed, and the rostellum is elongate and ribbon-like, while in *Lankesterella* the stigma is 2-lobed and the rostellum is subulate and elastic.

Autogamy is observed in these groups of orchids. Autogamous species are characterized by a reduction or lack of rostellum and viscidium. Good examples are *Eurystyles actinosophila* (Barb. Rodr.) Schltr., E. *borealis* A. H. Heller or *Lankesterella orthantha* (Kraenzl.) Garay. Autogamous taxa occur on the peripheries of ranges of particular genera, in Mesoamerica and in the north of South America in this instance. *Synanthes* was described by Burns-Balogh & Bernhardt in 1985 as a genus closely related to *Eurystyles*; those genera differ in gynostemium structure. The new genus was distinguished as a new taxon on the basis of studies of autogamous species only: *Synanthes borealis* (Heller) Burns-Bal., H.Rob. & Mercedes S. Foster, and *S. bertonii* Burns-Bal., H.Rob. & Mercedes S. Foster.

The subtribe Spiranthinae includes taxa with a relatively primitive type of gynostemium. The rostellum is soft, short to elongate; the viscidium is produced from the adaxial layer cells of the rostellum,

while the rostellum remnant is deeply notched or foveolate. Members of Stenorrhynchidinae are characterized by a sheath-like rigid viscidium, which is produced of the outer layer of rostellum cells, and the rostellum remnant is subulate. Stenorrhynchidinae are characterized by filiform caudiculae, distinctly thin at the end, and they are absent or very poorly developed in Spiranthinae. Both subtribes are among the most controversial groups of Orchidales. They were examined in detail by Balogh (1979), Garay (1982), Greenwood (1982), Balogh (1982), and Szlachetko (1991a, 1991b; 1992a-e; 1993a-e; 1994a-c). Thus both subtribes as delimited by Szlachetko require further detailed morphological studies and more extensive sampling of taxa for phylogenetic analysis.

Prescottiinae (clade D)

Our results show that Prescottiinae are a monophyletic group with high bootstrap support (BP 100). Because we were not able to extend the sampling of taxa for this subtribe, only representatives of the genus *Prescottia* were included in the analysis. Thus the observed monophyly seems to be of low reliability. Moreover, the results reported by Salazar *et al*. (2003) indicated that Prescottiinae are paraphyletic within Cranichidinae and Spiranthinae *sensu* Dressler. Thus the subtribe requires further studies and extensive sampling strategy in future phylogenetic examination.

4. General conclusions

The results of our phylogenetic analysis, based on examination of the ITS region, confirm the monophyly and separation of the subtribe Cyclopogoninae, as delimited by Szlachetko (1995). The unusual occurrence of *Odontorrhynchos variabilis* within Cyclopogoninae is not supported by morphological data, and, in our opinion, is probably a result of sampling error, due to an incorrect determination of source material. The Stenorrhynchidinae and Spiranthinae subtribes, as delimited by Szlachetko (1995), remain polyphyletic and thus further evaluation of subtribal concepts is required (see also Salazar *et al*. 2003). The clade Prescottiinae is strongly supported as monophyletic, although a lack of extensive sampling of taxa limits the reliability of this finding.

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