A reassessment of the phylogeny and circumscription of *Zaluzianskya*
(Scrophulariaceae)

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Abstract The genus *Zaluzianskya* (Scrophulariaceae s.s.) encompasses a diversity of traits, such as flowers ranging from 4 to 60 mm in length with various patterns of color and indumentum. However, this diversity, as described by the current taxonomic circumscription of *Zaluzianskya*, is an underestimate. The boundaries of this genus require expansion via incorporation of species from other genera and recognition of unnamed species. This study advances prior molecular phylogenies for the southern African genus through the addition of DNA regions and an up to 70% increase in population sampling, targeting specific taxa. Results are presented from analyses of five DNA regions, including nuclear ITS and four rapidly evolving chloroplast regions (*trnL-trnF*, *rpl16*, *rps16*, and *trnS-trnfM*). Our primary result is that several species of *Phyllopodium* should be included within *Zaluzianskya*, but that other species of *Phyllopodium* group with *Polycarena*, indicating the need for further phylogenetic work on these genera. Preliminary support for the incorporation of *Reyemia* into *Zaluzianskya* is reinforced here by the first molecular analysis to include both species of *Reyemia* and a strong sampling of species across *Zaluzianskya* and tribe Limoselleae. Two disjunct species of *Zaluzianskya* are also confirmed as members of this genus. Finally, a broad sampling of 21 populations of *Z. microsiphon* establishes their phylogenetic division into two to five separate lineages. Hybridization, coevolution, and cryptic speciation may each play a role in the evolution of *Z. microsiphon*. Further resolution within a clade comprising sections *Nycterinia* and *Macrocalyx* is needed to better understand their relationships.

Keywords: Disjunct distribution; *Phyllopodium*; *Reyemia*; Scrophulariaceae; South
; *Zaluzianskya*

1. Introduction

With a distribution centered in South Africa and including the botanical hotspot of the Cape Floristic Region (Cowling et al., 2009), the ca. 58 species of *Zaluzianskya* F.W. Schmidt (Scrophulariaceae, tribe Limoselleae; Table 1) provide a compact sample of morphological and ecological diversity, including variation in traits such as floral form, life history, and habitat preference (Hilliard, 1994; Manning and Goldblatt, 2010). For example, species include day-flowering, minute annuals of desert habitats and night-flowering shrublets of moist high-elevation meadows (Fig. 1). This range of characteristics allows study of various evolutionary questions, including those related to morphological evolution (Archibald et al., 2005), hybridization (Archibald et al., 2004; Campbell et al., 2016), coevolution (Anderson and Johnson, 2008), and more general aspects of pollinator-driven floral evolution (Anderson and Johnson, 2006; Anderson et al., 2005; Johnson et al., 2002).

The diversity of the *Zaluzianskya* lineage appears to be greater than originally estimated. Phylogenetic data provided preliminary support for inclusion of species from other genera such as *Reyemia* (Archibald et al., 2005; Kornhall et al., 2001; Manning & Goldblatt 2014); a new species of *Zaluzianskya* was recently described by Manning and

Goldblatt (2010); and polyphyly in molecular phylogenies suggests the possibility of unrecognized cryptic species, particularly within *Z. microsiphon* (Archibald et al., 2005). However, prior studies relating to the placement of *Reyemia* only included a sample of one of the two species and either had low levels of sampling within *Zaluzianskya* (Kornhall et al., 2001) or relatively low sampling across tribe Limoselleae (Archibald et al., 2005), both of which are important for a thorough assessment of the placement of this species. Confirmation of the polyphyly of *Z. microsiphon* also requires greater sampling, with the prior phylogenies only including three populations (Archibald et al., 2005). In addition to these potential expansions of *Zaluzianskya*, the relationships of two species of *Zaluzianskya* that are geographically disjunct from the remainder of the genus have not been tested phylogenetically.

Tribe Limoselleae sensu Kornhall and Bremer (2004) includes *Limosella* and ca. 25 genera formerly placed in tribes Manuleeae and Selagineae (Hilliard, 1994, 1999; Kornhall and Bremer, 2004). Hilliard (1994, 1999) provided monographs for many of these species. *Reyemia* was described by Hilliard (1992) to accommodate *Zaluzianskya nemesioides* Diels plus the new *R. chasmanthiflora* Hilliard, but later molecular analyses suggested that the species were nested within *Zaluzianskya*. In a tribal-level study, Kornhall et al. (2001) found preliminary evidence that *Reyemia chasmanthiflora* was nested within *Zaluzianskya* but only included three species of *Zaluzianskya*. Archibald et al. (2005) increased sampling in the genus to 23 species to produce the first inference of phylogeny across all sections of *Zaluzianskya*. They found strong support for the nesting of *R. nemesioides* within *Zaluzianskya* (99% bootstrap, BSt), but did not sample *R. chasmanthiflora* and did not have the extensive sampling of other genera in Limoselleae

of Kornhall et al. (2001). These findings, and the morphological affinity between the two genera, led Manning and Goldblatt (2014) to merge *Reyemia* back into *Zaluzianskya* as sect. *Reyemia*. Our present study retests the phylogenetic placement of *Reyemia*, including both species of the genus and a broad sampling of species across both *Zaluzianskya* and Limoselleae. In addition, preliminary analyses indicated that species of *Phyllopodium* (Limoselleae) are also nested in *Zaluzianskya* (Archibald unpub.). In the present study, DNA sequences from seven of the 26 species of *Phyllopodium* allowed a first examination of this possibility. We also expanded our outgroup sampling to include all of the major clades of tribe Limoselleae.

Zaluzianskya is a component of many major biomes in South Africa and extends into the neighboring countries of Lesotho, Swaziland, and Namibia. Only two species occur elsewhere (Hilliard, 1994). One of these, *Zaluzianskya tropicalis* (sect. *Nycterinia*), is widespread in Zimbabwe and reaches into Mozambique, while the second, *Zaluzianskya elgonensis* (sect. *Zaluzianskya* subsect. *Noctiflora*), is widely disjunct from its allies. Seemingly known only from two collections, on Mt. Elgon in Uganda and Mt. Kilimanjaro in Tanzania, *Z. elgonensis* is geographically separated from the rest of its subsection by at least 3000 km (Hilliard, 1994). Neither of the disjunct species was included in previous molecular phylogenetic analyses, and the biogeographical factors behind their isolated distributions are unknown. Both species were included in the present study.

Prior molecular phylogenetic analyses of *Zaluzianskya* inferred three major clades (Archibald et al., 2005). Clades 1 and 2 each included members of sections *Zaluzianskya* and *Holomeria*, with clade 2 also including *Reyemia*. Relationships within clade 3, or the

Nycterinia + *Macrocalyx* clade, remained obscure, with incomplete resolution and some conflict between gene trees. Johnson et al. (2002) produced a morphological phylogeny for *Zaluzianskya* sect. *Nycterinia*, but with generally weak bootstrap support across the clade. In the molecular phylogenies, clade 3 included all sampled members of sect. *Nycterinia* and the single sampled representative of sect. *Macrocalyx*. Populations of one species of clade 3, *Z. microsiphon*, were broadly separated in these analyses. Chloroplast and ITS data differed in resolution of this clade, with ITS showing no well-supported resolution beyond a division of *Z. microsiphon* and a strongly-supported sister relationship between the two sections (BSt=70). The chloroplast tree divided clade 3 into two subclades, one of which included *Z. mirabilis* (from sect. *Macrocalyx*), one of three sampled populations of *Z. microsiphon*, and two other species from sect. *Nycterinia* (BSt=99). Sampling of *Z. microsiphon* in the present study has been increased from 3 to 21 populations from across its geographic range in Lesotho and southeastern South Africa (including parts of KwaZulu-Natal, Eastern Cape, Free State, and Mpumalanga Provinces). This allowed more thorough tests of the potential division of this species.

A robust phylogenetic framework is important for resolving the circumscription of the *Zaluzianskya* lineage and for investigating evolutionary patterns and processes. Archibald et al. (2005) sequenced ITS, *trnL-trnF*, and *rpl16* across the genus. We have augmented those molecular phylogenetic hypotheses with the addition of two gene regions (*rps16* and *trnS-trnfM*) and key taxa and populations. We address three primary questions to improve our understanding of diversity in the *Zaluzianskya* clade: 1) Is there strong phylogenetic support for the inclusion of both former species of *Reyemia* and some members of *Phyllopodium* in *Zaluzianskya*? 2) How are disjunct species of

Zaluzianskya related to other members of the genus? 3) Are multiple, cryptic species revealed within *Z. microsiphon* when many populations are sampled across its range?

2. Materials and methods

2.1. Taxon collection and sequencing

Fresh leaf material (ca. 20 mg per individual) was collected on silica gel from each newly collected population; voucher information is given in the Appendix. Initially, 116 populations from 46 species were analyzed. This included 99 populations from 29 species of *Zaluzianskya*, with representatives from each subsection of the genus and both members of sect. *Reyemia*. Only populations that were successfully sequenced for at least three of the five DNA regions were retained for further analyses (but see discussion below on level 3 analyses). Samples identified as putative hybrids or introgressants by Archibald et al. (2004) were also removed to minimize the potentially confounding influence of horizontal gene flow on phylogenetic inference (McDade, 1992; Soltis et al., 2008). Of the remaining accessions, 52 populations had never previously been sampled in a published phylogeny (see bold accessions in the Appendix). Within *Zaluzianskya*, 29 species were included, with 2 or 3 populations of most species and 21 populations of *Z. microsiphon*. The two disjunct species were included via DNA extractions done at Kew from herbarium specimens collected in 1966 (*Z. tropicalis*) and 1970 (*Z. elgonensis*). Four species were sequenced from *Phyllopodium*, with three other species (four populations) of that genus added using GenBank sequences (indicated by "GB").

Outgroups were chosen based on their phylogenetic proximity to *Zaluzianskya* in Archibald et al. (2005) and in broader analyses in Scrophulariaceae (Kornhall, 2004; Kornhall et al., 2001).

Further analyses were primarily conducted at three main levels (Table 2). The sampling within *Zaluzianskya* was similar across these analyses, while the outgroup (OG) sampling varied greatly. Level 1 focused on the *Zaluzianskya* clade and a few key OGs for five loci. Level 2 included more OG populations but more missing data across the five loci. Level 3 added GenBank sequences to include 23 of the 27 genera of Limoselleae but with fewer (two) loci. Additional analyses were run to determine the influence of key samples with missing data. For example, analyses were run with and without *Phyllopodium mimetes* (only sequenced for ITS and *trnL-trnF*, but one of three sampled species of *Phyllopodium* that are placed within the *Zaluzianskya* clade) and *Trieenea taylorii* (only sequenced for ITS, but noted in the Discussion for sharing a number of traits with *Zaluzianskya*). The inclusion or exclusion of these two taxa did not alter other relationships, and so results are shown with them included. GenBank sequences for *Selago canescens* came from two individuals listed as GB1 and GB2 in the Appendix. However, initial analyses indicated that each was placed in the same location on the tree, and so they were concatenated in final analyses (indicated as GB12).

Sequencing methods followed those used previously (Archibald et al., 2005). Newly collected DNA was extracted from leaf samples using standard CTAB methods (Doyle and Doyle, 1987; Mort et al., 2001). Sequences were produced for the nrDNA internal transcribed spacer regions (ITS-1 – 5.8S – ITS-2; see Baldwin et al., 1995), and four regions from the chloroplast genome: the *trnL* intron, *trnL* exon, and *trnL-trnF*

spacer region (hereafter referred to as the *trnL-F* region), the *rpl16* intron, the *rps16* intron, and the *trnS-trnfM* intergenic spacer (*trnS-fM*). For discussion of the utility of these and other rapidly-evolving cpDNA regions see Shaw et al. (2005; 2007) and Mort et al. (2007). Standard primers were employed for PCR: N-nc18S10 and C26A for ITS (Wen and Zimmer, 1996), B49317 (“c”) and A50272 (“f”) for *trnL-F* (Taberlet et al., 1991), L16 exon1 and L16 exon2 for *rpl16* (Downie et al., 2000), rpS16F and rpS16R for *rps16* (Oxelman et al., 1997; Shaw et al., 2005), and trnSUGA and trnfMCAU for *trnS-fM* (Demesure et al., 1995; Shaw et al., 2005).

PCR amplifications (25 µl) consisted of 1 µl DNA, 0.2 µM of each primer, 3 mM MgCl₂, and 1X Bullseye Taq DNA Polymerase Mix (150 mM Tris-HCl pH 8.5, 40 mM (NH₄)₂SO₄, 0.2% Tween 20, 0.4 mM dNTPs, 0.5 units/µL Bullseye Taq polymerase, and stabilizer; Midsci, Saint Louis, Missouri, USA). A MyCycler (Bio-Rad, Hercules, California, USA) thermocycler was used with the following settings: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; 10 min at 72°C. Each thermocycler run included a negative control reaction of 25 µl with all of the reagents except for the DNA. PCR products were either purified using a PEG clean-up protocol (Soltis and Soltis, 1997) and sequenced by MacroGen, Inc. (Seoul, South Korea), or sent to htSEQ (High-Throughput Genomics Unit, University of Washington, USA) for ExoSAP purification and sequencing. In both cases, sequencing was completed with the same primers as used for PCR amplification.

2.2. Phylogenetic analyses and hypothesis testing

Individual contigs were edited, assembled, and aligned using Sequencher (GeneCodes Inc., Ann Arbor, Michigan) and Se-Al v. 2.0a11 (Rambaut, 2002); or Geneious v. 6.1.7 (Biomatters, Auckland, New Zealand) with alignments using MUSCLE settings and adjustments by eye. Gaps were coded as characters using a modified version of the complex indel coding method of Simmons & Ochoterena (2000), as implemented by SeqState v. 1.32 (IndelCoder: modified complex coding option, Müller, 2005). All chloroplast sequences were concatenated into one data matrix a priori, and the chloroplast and ITS datasets were analyzed both separately and concatenated with maximum parsimony (MP), maximum likelihood (ML), and Bayesian Inference (BI).

Parsimony analyses were conducted in PAUP* v. 4.0b10-x86 (Swofford, 2003) with the heuristic search option, MulTrees, no MaxTrees limit, swapping on all trees, and parsimony options set to collapse branches if minimum length is zero (“amb-”). Uninformative characters were excluded before all analyses and all remaining characters were equally weighted and unordered. One thousand quick searches with random taxon addition and NNI branch swapping were used to locate multiple islands of minimum-length trees, forming a starting pool of trees for more thorough searches employing tree bisection-reconnection (TBR; Maddison, 1991). Separate TBR searches were run in different putative islands and inferred consistent relationships. Relative support for clades was assessed via jackknife analyses (JK; 5000 replicates). PAUP* was set to emulate Jac resampling with character deletion at 37%. For the concatenated nuclear and chloroplast dataset, analyses were run using both nucleotide and indel characters, nucleotide characters alone, and indel characters alone, for comparison. Results indicated consistent signal between the two types of characters, with one exception that will be discussed.

Otherwise, only the combined nucleotide and indel analyses will be discussed here.

Separate analyses of data from each genome also included both nucleotide and indel characters unless noted otherwise.

Maximum likelihood and BI analyses were conducted on the nucleotide datasets. Given the similarity of their results, only BI analyses will be discussed. Model parameters were estimated using the Akaike Information Criterion (AIC) of jModelTest v. 2.1.3 (Posada, 2008). Bayesian analyses were conducted in MrBayes v. 3.2.1 (Ronquist et al., 2012) and were partitioned by gene region, allowing independent evolutionary models for each. Two runs of metropolis-coupled Markov chain Monte Carlo simulations each used four linked chains (three heated and one cold) and default priors for all model parameters. The final analyses were halted when the average standard deviation of split frequencies was below 0.001. After a burn-in of 25%, parameter values were sampled every 10,000 generations. Convergence was assessed using Tracer v. 1.6 (Rambaut and Drummond, 2007) and AWTY (Nylander et al., 2008; Wilgenbusch et al., 2004; "compare" and "cumulative" utilities).

Topological incongruence was assessed by visually comparing the well-supported nodes from each analysis, considering nodes with at least 70% jackknife support (see Kellogg et al., 1996; Mason-Gamer and Kellogg, 1996) or 0.95 posterior probability (Simmons, 2004). In general, any support values at those levels or above are considered “strong” in this paper. Alternate phylogenetic hypotheses were tested using approximately unbiased (AU) tests in Consel (Shimodaira, 2002), using the concatenated level 1 and level 2 datasets. Candidate trees for AU tests were produced in Garli v. 0.96 (Zwickl, 2006) via unconstrained ML and bootstrap analyses (500 replicates) as well as

those constrained to include or exclude clades of interest (100 – 500 replicates).

Conclusions were consistent across multiple runs of Consel with differing candidate tree sets. Final AU p-values are from runs using all candidate trees from unconstrained and constrained ML and bootstrap analyses.

3. Results

In most cases, analyses produced concordant results regardless of optimality criterion (MP or BI), DNA loci (concatenated or separate), or taxon sampling (levels 1, 2, or 3); exceptions will be noted. Dataset characteristics are described in Table 2. Minimal effective sample sizes were greater than 700 for each parameter in all BI analyses and potential scale reduction factors were all 1.000. The identification of three main clades within *Zaluzianskya* (clades 1, 2, and 3) and the relationships among them were as found by Archibald et al. (2005), with some complications regarding clade 2 explained below. Figure 2 summarizes the inferred evolutionary relationships and clade support among *Zaluzianskya* and outgroups, while Fig. 3 focuses on phylogenetic relationships within *Zaluzianskya*.

3.1. Relationships among genera and placement of other genera within *Zaluzianskya*

All phylogenetic analyses placed the monophyletic sect. *Reyemia* within *Zaluzianskya* with strong support. Its connection with clade 2 was supported or not contradicted by all analyses, with weakest support from cpDNA analyses. Three species of *Phyllopodium* were also strongly placed within the *Zaluzianskya* clade, whereas the

other four sampled species of *Phyllopodium* were placed in the outgroup near *Polycarena*. Only level 3 analyses included all three of the species of *Phyllopodium* that nest within *Zaluzianskya*, and they resolved *Ph. mimetes* and *Ph. cf. cephalophorum* as sister with strong support, with these two species in turn weakly to strongly sister to the remainder of clade 2. *Phyllopodium cordatum* was strongly resolved as sister to clade 2 + 3 by concatenated and most ITS analyses but in a polytomy with clades 2 and 3 (and *Ph. mimetes*, if sampled) by cpDNA analyses. In contrast, the MP-ITS analyses with low outgroup (OG) sampling weakly to moderately inferred *Ph. cordatum* as sister to the remainder of the *Zaluzianskya* clade. Bayesian analyses of those ITS datasets or MP-ITS analyses without indel characters instead placed *Ph. cordatum* within the *Zaluzianskya* clade. Primarily one indel character seems to give the weak support for excluding *Ph. cordatum* from the rest of this clade. The signal across ITS and certainly in combination with cpDNA characters nests *Ph. cordatum* deeper within *Zaluzianskya*. Regardless, all analyses showed that *Ph. cordatum* groups with *Zaluzianskya* rather than with the species of *Phyllopodium* that are in the OG. A monophyletic *Zaluzianskya* was rejected by AU tests for all but 3-5% of the trees. In the rare occasions when it was accepted by AU tests while monophyletic, *Zaluzianskya* s.s. (i.e., not including members of *Reyemia* or *Phyllopodium*) was sister to the other typical members of the *Zaluzianskya* clade and placed on a short branch. The monophyly of *Phyllopodium* s.l. was completely rejected by AU tests (max p-value <0.001).

The strongly supported clade 2 of *Zaluzianskya* in Archibald et al. (2005) is now associated with several newly sampled accessions, including three *Phyllopodium* species plus an additional species of sect. *Reyemia*. This portion of the phylogeny includes four

strongly supported clades: *Z. peduncularis* + *Z. cohabitans* + *Z. elgonensis*, *Z. divaricata* + *Z. pusilla*, sect. *Reyemia*, and *Ph. mimetes* + *Ph. cf. cephalophorum* (for the analyses that included them). The relationships among these clades varied somewhat or were unresolved, with the *Reyemia* clade and *Ph. mimetes* changing positions. Support for these alternate placements was often weak or lacking. More evidence is needed to determine basal relationships within clade 2 and to verify whether *Ph. mimetes* and *Ph. cf. cephalophorum* belong with this clade or elsewhere within *Zaluzianskya*.

Up to four other species of *Phyllopodium* were sampled for some analyses, and most analyses placed them in a clade with up to six species of *Polycarena*. The exception is the level 3 cpDNA analyses, which placed some or all of these *Phyllopodium* species in a polytomy with *Polycarena* and other OGs. *Polycarena* was strongly supported as monophyletic by all analyses but was nested within *Phyllopodium* by most. It was weakly to strongly inferred as sister to *Ph. cuneifolium* by ITS and concatenated analyses, and weakly inferred as sister to *Ph. rustii* and *Ph. dolomiticum* by some cpDNA analyses. Other cpDNA analyses did not resolve relationships between the *Polycarena* clade and *Phyllopodium*.

In general, relationships among the remaining OGs are consistent with those found by Kornhall and colleagues (Kornhall, 2004; Kornhall and Bremer, 2004; Kornhall et al., 2001), and those studies should be referred to for more detailed discussion for many of those clades. The OG accessions that were sequenced by our research group do not have "GB" in their population ID (Fig. 2) and, for the most part, are placed with other species of their genera. One exception is *Trieneea*, which was not monophyletic in these trees. However, only our ITS sequence is available for *T. taylorii*, and while BI analyses

strongly supported it as sister to *Pseudoselago*, MP analyses placed it unresolved in a position potentially closer to *T. glutinosa* GB. There was mixed support in our analyses for non-monophyly in *Lyperia*, *Hebenstretia*, and *Selago*, but only *Phyllopodium* in the outgroups was strongly and consistently resolved as polyphyletic. See Kornhall (2004, Ch. 4) in particular for relationships within *Selago* and close relatives. Overall, some differences arose among our analyses at different taxon-sampling levels, but this is unsurprising given the range from four to 48 accessions within the OG clades, and it does not result in any hard incongruences. In the OG, only the relationships among *Manulea*, *Sutera*, and *Chaenostoma* showed conflict between the ITS and cpDNA level 3 analyses. Regardless, these phylogenies are consistent with the decision of Kornhall and Bremer (2004) to divide *Sutera* s.l. into two genera (*Sutera* s.s. and *Chaenostoma*): cpDNA support a *Manulea* + *Chaenostoma* clade while ITS supports a *Manulea* + *Sutera* s.s. clade.

3.2. Relationships in *Zaluzianskya*, with focus on clade 3, the *Nycterinia* + *Macrocalyx* clade

Clade 3 was resolved as monophyletic by all analyses except some MP-cpDNA analyses, which tentatively excluded *Z. tropicalis* from this clade. In the latter, *Z. tropicalis* was placed either in a polytomy with the rest of clade 3 and several other lineages or weakly as sister to sect. *Reyemia*. However, the differing MP-cpDNA resolutions had low clade support, and the cpDNA data for *Z. tropicalis* was relatively weak: it is the only accession of *Zaluzianskya* for which two of the four cpDNA regions failed to sequence. Concatenated analyses strongly support the monophyly of clade 3 and

the placement of *Z. tropicalis* sister to the rest of the clade, while any conflicting placement by analyses of individual loci is not well supported (Fig. 3).

The only subclade in clade 3 that had strong support from all analyses was the clade of two populations of *Z. mirabilis* (i.e., the sole species included from sect. *Macrocalyx*). Many relationships were unresolved by all analyses. Most clades that did receive high support in some analyses were not strongly inferred by others (ITS in particular). There was one case of strong conflict between ITS and cpDNA. As also noted by Archibald et al. (2005), *Z. mirabilis* was very strongly inferred as nested in clade 3 by cpDNA and concatenated analyses, but was well supported as sister to a monophyletic sect. *Nycterinia* by MP-ITS analyses (JK = 72, level 1). Contrary to those results, the BI-ITS analyses do not resolve a monophyletic sect. *Nycterinia*, instead having no support for basal nodes within clade 3. The monophyly of sect. *Nycterinia* was neither supported nor rejected by AU tests.

When analyzed alone, ITS failed to resolve relationships in most of clade 3, only consistently inferring the clade of two populations of *Z. mirabilis*. Otherwise, the MP-ITS analyses had just two well-supported clades, a sect. *Nycterinia* clade, noted above, and a clade of four populations of *Z. microsiphon* (i.e., *Z. microsiphon*-3, A14, A15, A16; JK = 78, level 1). The latter is designated here *Z. microsiphon*-S, due to the relatively southern distribution of those populations compared to other sampled populations of *Z. microsiphon*. Within clade 3, the BI-ITS analyses only had strong support (PP>0.95) for the clade of *Z. mirabilis* populations. In contrast, the cpDNA and concatenated analyses strongly inferred a basal division of clade 3 (excluding *Z. tropicalis*) into two well-supported clades (clades A and B; Fig. 3). They nested *Z. mirabilis* (of sect. *Macrocalyx*)

in clade 3A along with *Z. microsiphon*-S and two populations each of *Z. angustifolia* and *Z. maritima*. The cpDNA and concatenated analyses also strongly supported multiple subclades within clades A and B. *Zaluzianskya capensis* was strongly inferred as monophyletic, as was *Z. glareosa*, while the monophyly of many other species remained uncertain or contradicted by these analyses. *Zaluzianskya microsiphon*, in particular, was divided into multiple clades that did not group together on any of the trees. The monophyly of *Z. microsiphon* was rejected by AU tests (max p-value <0.001).

Clade 2 was discussed above in the context of generic relationships. Although many relationships in clade 1 are well supported, there are some conflicts between the ITS and cpDNA analyses due primarily to the unstable placement of several key taxa. In particular, *Z. affinis*-1 moved between two separated clades of *Z. affinis*, and *Z. benthamiana*-1 was sometimes placed within the *Z. violacea* clade. These differences are strongly supported by at least one analysis. Level 3 ITS analyses included one additional GenBank accession for both *Z. gracilis* and *Z. villosa* that were strongly inferred as members of the *Z. villosa* + *Z. affinis* + *Z. gracilis* clade.

4. Discussion

These analyses establish that the diversity within the *Zaluzianskya* clade is greater than previously known. A lack of monophyly for *Zaluzianskya* and *Phyllopodium* is revealed, with some species of the latter and all species of *Reyemia* now clearly shown as members of the *Zaluzianskya* clade. Two species of *Zaluzianskya* that are geographically isolated from the remainder of the genus were included in a molecular phylogeny for the

first time and are confirmed as belonging in this genus. Finally, independent lineages are established among 21 populations of "*Z. microsiphon*," supporting the presence of multiple, rapidly evolving, species under this single name. Each of these results will be discussed in more detail below.

4.1. Polyphyly of *Phyllopodium* and its partial inclusion with *Reyemia* in the *Zaluzianskya* clade

Our phylogenetic analyses demonstrate that *Zaluzianskya* s.s. is not monophyletic, due to the embedding of species from at least two other genera. Despite evident differences in floral morphology, the transfer of *Reyemia nemesioides* and *R. chasmanthiflora* into *Zaluzianskya* now has strong phylogenetic support (Figs. 2, 3). Resupination, or developmental turning of a flower so that it is effectively upside down at maturity, is unique to *Reyemia* within tribe Limoselleae. These two species also add more highly branched inflorescences and presence of hairs in the corolla throat to the floral variation found in *Zaluzianskya*. See Manning and Goldblatt (2014), Archibald et al. (2005), and Hilliard (1994) for further discussion of the morphology and relationship of these two genera. The AU tests do not absolutely exclude the possibility of a monophyletic *Zaluzianskya* s.s., but they do fully reject a monophyletic *Phyllopodium*. All analyses demonstrate the need for detailed phylogenetic analyses of the latter genus. Only 4 to 7 of the 26 species of *Phyllopodium* were sampled, but our analyses segregate them into at least two, and up to four or five, separate groups. Available data place *Ph. cordatum*, *Ph. mimetes*, and *Ph. cf. cephalophorum* in the *Zaluzianskya* clade, likely grouped with clades 2 and 3. The other species of *Phyllopodium* are placed as outgroups,

either in a grade sister to *Polycarena* or at least partially unresolved within a larger clade containing *Polycarena* and other OGs.

We know of no broad phylogenetic studies of *Phyllopodium*. The studies of Kornhall and colleagues (Kornhall, 2004; Kornhall and Bremer, 2004; Kornhall et al., 2001) sampled up to four species of *Phyllopodium* (*Ph. cuneifolium*, *Ph. dolomiticum*, *Ph. multifolium*, *Ph. cf. cephalophorum*), up to five of *Polycarena*, and up to four of *Zaluzianskya* (*Z. capensis*, *Z. glareosa*, *Z. gracilis*, *Z. villosa* for ITS, Kornhall, 2004; *Z. benthamiana*, *Z. katherinae*, *Z. glareosa* for *ndhF*, Kornhall et al., 2001). The focus of those studies ranged from multiple tribes to subsets of genera in tribe Limoselleae. In all cases, most of *Phyllopodium* forms a clade with *Polycarena*, sometimes as sister genera and sometimes as a grade, as seen in the outgroup of the present study. *Phyllopodium cf. cephalophorum* is placed with *Zaluzianskya*, but that accession was misidentified in those papers as *Z. minima*, and so the odd placement was not noticeable. Once it was added to our analyses, with taxon sampling focused within *Zaluzianskya*, we noted its placement was not consistent with *Z. minima* and identified it as a member of subgenus *Leiospermum* in *Phyllopodium* based on examination of the original specimen. That subgenus includes four species. Our determination is that this specimen is probably *Ph. cephalophorum*, but could be *Ph. phyllopodioides*. Hilliard (1994) recognized these as separate species, but also stated (p. 458) "The two species are very closely allied and it is difficult to formulate the distinctions between them."

A number of Hilliard's "principal characters" for the genera in the tribe are shared by *Zaluzianskya* and *Phyllopodium* (see Table 1 of Hilliard, 1994). In both genera the floral bract is adnate to the calyx, stem hairs are eglandular or stalked glandular,

inflorescences are not cymes, posticous stamen filaments are decurrent to the corolla, stigmas are ligulate, and a lateral gland nectary is present. In the species for which chromosome counts are available, *Zaluzianskya* (nine species counted) and *Phyllopodium* (three species) also have the same number of chromosomes: $2n=12$. Other species in the tribe for which counts are available range from $2n=12$ to 28. This combination of features is only additionally shared by three other genera of the 17 in the tribe (sensu Hilliard, 1994): *Reyemia*, *Melanospermum*, and *Trieneea*. However, *Trieneea* has chromosome counts of both 12 and 14, and counts are not available for *Reyemia* and *Melanospermum*. Both *Melanospermum* and *Trieneea* are placed separately from *Zaluzianskya/Reyemia* in our analyses (Fig. 2) and the tribal-level phylogenies of Kornhall et al. (2001). Some of these shared traits appear to be plesiomorphic (Kornhall et al., 2001). Regardless, a more thorough sampling of *Phyllopodium* for phylogenetic analyses is needed before synapomorphic characters for the *Zaluzianskya* clade can be determined and taxonomic changes made.

Morphologically, species of *Zaluzianskya/Reyemia* are distinguished from those of *Phyllopodium* by their toothed, plicate calyx whereas calyces of *Phyllopodium* are lobed halfway or nearly to the base. Calyx shape is diagnostic for multiple taxa in Hilliard's (1994) monograph. Anther number and placement for *Phyllopodium* is also different from *Zaluzianskya*, although the latter is variable for these traits. Most species of *Phyllopodium* have four, exserted anthers (two or three in selfing species). *Zaluzianskya* has two or four anthers, either all included or only the anticous pair exserted (although rarely the posticous pair is partly or entirely exserted as well); and both species of *Reyemia* have two anthers that are fully exserted.

Hilliard (1994) divided *Phyllopodium* into two subgenera: *Phyllopodium* (22 species) and *Leiospermum* (four species; Table 1). The four OG-*Phyllopodium* species are from subgenus *Phyllopodium* subgroups 1A (*Ph. cuneifolium*, *Ph. rustii*) and 1B (*Ph. dolomiticum*, *Ph. multifolium*). The three sampled species that were placed with *Zaluzianskya* are from subgroup 2A (*Ph. cordatum*, *Ph. mimetes*) and subgenus *Leiospermum* (*Ph. cf. cephalophorum*). With three species from putatively divergent groups within *Phyllopodium* being strongly supported as nested within *Zaluzianskya*, it is likely that new combinations will be needed for those and other species from *Phyllopodium*. Taxonomic changes have not been made here, pending the collection of more evidence for each individual species whose movement is proposed. Groups 1 and 2 of *Phyllopodium* are mainly distinguished by Hilliard (1994) based on minute characteristics of the seed coat observed under an SEM, but she also discusses larger features for each group. For example, group 1 differs from 2 in having elongated racemes versus usually compact round heads. The latter is also characteristic of subgen. *Leiospermum*, and thus links all sampled *Phyllopodium* species that are placed in the *Zaluzianskya* clade. This is not a trait that would be expected to link outside species with *Zaluzianskya*, given that *Zaluzianskya* species have terminal spikes (or loose panicles, in the case of sect. *Reyemia*). Thus, the addition of both sect. *Reyemia* and these species of *Phyllopodium* to the *Zaluzianskya* clade significantly alters our understanding of the evolution of inflorescence architecture within this group. These species of *Phyllopodium* also differ from *Zaluzianskya* s.s. in having a beard of clavate hairs on the posticous lip of the corolla. This feature is found in a number of genera within the tribe, including *Reyemia*. It is not found in *Zaluzianskya* s.s., although some species have clavate hairs in

a ring around the throat of the corolla (Hilliard, 1994). These corolla hairs have been hypothesized to influence pollinator interactions (Johnson et al., 2002).

We included 6 of the 17 species of *Polycarena*, with multiple members from both of the taxonomic groups proposed for this genus by Hilliard (1994). These species formed a very well-supported clade, but there is strong support for the nesting of this clade within a *Phyllopodium* clade by some of our analyses. Other analyses place *Polycarena* in a polytomy with some or all OG-*Phyllopodium*. Regardless, a close relationship between *Polycarena* and a subset of *Phyllopodium* is clear. This relationship is unsurprisingly consistent with Kornhall (2004), the source of our GenBank sequences, and has been noted since the naming of these genera (Bentham 1836). Some workers have suggested combining them (as noted by Hilliard, 1994, p. 71-72). However, Hilliard (1994) gives a number of features that may distinguish the two genera, including presence/absence of eglandular hairs and wings on seeds. Thorough phylogenetic investigations are needed to identify true synapomorphic characters and reconcile inferred genetic and morphological groups.

4.2. Placement of disjuncts and relatives within *Zaluzianskya*

While *Zaluzianskya* s.s. is found throughout most of South Africa and extends into several nearby countries, *Phyllopodium* is confined to the western and southern parts of South Africa, extending somewhat into Namibia. *Zaluzianskya* sect. *Reyemia* has an even narrower distribution, occurring only in a small area of west-central South Africa. Thus, these additions to the *Zaluzianskya* clade play no role in bridging the distributional gap seen between the rest of *Zaluzianskya* and the two disjunct species: *Z. elgonensis*

from Tanzania and Uganda and *Z. tropicalis* from Zimbabwe and Mozambique.

Nonetheless, in the phylogeny, the latter two species were placed approximately where expected based on their taxonomic classification.

The addition of *Z. elgonensis* increased the number of sampled species from sect. *Zaluzianskya* subsect. *Noctiflora* to two of four (including also *Z. peduncularis*), allowing the first tests of the subsection's monophyly. These two do form a strongly supported subclade of clade 2, but only with the addition of *Z. cohabitans* of sect. *Zaluzianskya* subsect. *Zaluzianskya* (Fig. 3). The rest of subsect. *Zaluzianskya* is placed in clade 1. A sister relationship between *Z. peduncularis* and *Z. cohabitans* is strongly inferred by some analyses, while they form a polytomy with *Z. elgonensis* in others. *Zaluzianskya cohabitans* is distinct from other members of its subsection not only in its phylogenetic placement but also in its elliptic and typically entire petal lobes, compared to the notched petal lobes of most members of subsect. *Zaluzianskya*. Entire petal lobes characterize all members of clade 2, including the additions from *Reyemia* and *Phyllopodium*. This further supports the assessment by Archibald et al. (2005) that petal shape may be a useful character for infrageneric classification of *Zaluzianskya*.

Zaluzianskya tropicalis is generally resolved in clade 3 with the rest of section *Nycterinia*; the concatenated data strongly support a sister relationship between *Z. tropicalis* and the remainder of clade 3. Hilliard (1994) discusses *Z. tropicalis* as resembling *Z. glareosa* (two populations sampled here) and *Z. distans* (not sampled here). The phylogenies do not demonstrate a particular affinity between these taxa, but more data are needed. At a minimum, this disjunct species is strongly supported as a member of *Zaluzianskya*, but it seems to have diverged relatively early from the rest of its section.

4.3. The division of *Z. microsiphon* within the rapidly diversifying *Nycterinia* + *Macrocalyx* clade (clade 3)

The clade in *Zaluzianskya* that remains least well known in terms of phylogenetic relationships is clade 3 (Fig. 3). It is strongly supported by all analyses as a monophyletic group, with the unlikely but possible exclusion of *Z. tropicalis*. Close genetic ties between the 15 sampled species imply rapid diversification. In addition to a partial lack of resolution, there is some conflict between the ITS and cpDNA trees. MP-ITS analyses inferred a monophyletic sect. *Nycterinia* while the other analyses nested *Z. mirabilis* within sect. *Nycterinia* or were not informative. Notably, BI-ITS analyses were in the latter group, adding long-branch attraction as a possible explanation for the proposed conflict. This is consistent with the relatively long branch to *Z. mirabilis*. See Archibald et al. (2005) for further discussion of the potential inclusion of *Z. mirabilis* among species from sect. *Nycterinia*. Three species from sect. *Nycterinia* were added to this study but not available to Archibald et al. (2005): *Z. spathacea*, *Z. schmitziae*, and the disjunct *Z. tropicalis*. Consistent with their classification, they were all placed in clade 3.

A previously-noted phylogenetic division of the day-flowering *Z. microsiphon* (Archibald et al., 2005) is confirmed by these analyses with much greater population sampling. Concatenated and cpDNA analyses decisively separate *Z. microsiphon*, with four populations, designated as *Z. microsiphon*-S, nested in one well-supported subclade (3A) and the remainder scattered in the other (subclade 3B). *Zaluzianskya microsiphon*-S is also the only group in sect. *Nycterinia* that is strongly inferred both by some ITS and cpDNA analyses. The populations of *Z. microsiphon*-S are separated by a minimum of 85

km from the nearest sampled population of *Z. microsiphon* from subclade 3B. However, additional exploration of the intervening area is needed to check for other populations. There is weak to strong support for further subdivision of *Z. microsiphon* within clade 3B in cpDNA and concatenated trees, with populations grouping with members of other species. As many as six independent lineages of *Z. microsiphon* are strongly supported by one or more analysis, despite lack of resolution for many nodes in clade 3. Given the uncertain relationships within this clade, it is unsurprising that the exact pattern of evolution for these *Z. microsiphon* lineages is unclear. It is clear that the evolution of these plants has been more complex than recognized by previous workers (e.g., Hilliard, 1994). The relative importance of convergent evolution vs. hybridization in these divisions remains undetermined. If further data confirm the division into six or more lineages, it seems unlikely that each of those lineages was artificially separated on the tree due to the confounding effects of hybridization on phylogenetic analyses. Although *Z. microsiphon* is locally sympatric with multiple members of sect. *Nycterinia* (pers. obs.), the rest of the section is night flowering. Hybridization has been confirmed at one site between *Z. microsiphon* and *Z. natalensis*, demonstrating that the difference in flower-opening time is not an absolute barrier to gene flow in this case. However, it is at least a partial barrier, and it combines with other forms of reproductive isolation, such as differences in floral orientation that lead to differential pollinator attraction (Archibald et al., 2004; Campbell et al., 2016).

An important factor in the evolution of these lineages appears to be long-proboscid flies. *Prosoeca ganglbaueri* (Nemestrinidae) is the main pollinator of most populations of *Z. microsiphon*, and *Z. microsiphon* is the primary (although not the only)

food source for the flies (Anderson and Johnson, 2008; Johnson et al., 2002). Anderson and Johnson (2008) found trait covariation for corolla tube length and proboscis length, indicating coevolution or possibly sequential trait evolution. Variation in tube lengths within a population was relatively low, but average lengths across populations ranged widely from 19.4 to 54.6 mm (N = 20 - 59). Most of the populations studied by Anderson and Johnson (2008) are included here, allowing us to add a preliminary phylogenetic framework to their comparison. Many of the well-supported clades of *Z. microsiphon* encompass a variety of tube-lengths. For example, average tube lengths even just across populations of *Z. microsiphon*-S range from ca. 33 to 52 mm. This suggests that the rate of tube-length evolution is quite fast, with repeated changes within separate lineages of *Z. microsiphon*. However, again, hybridization may also be at work. According to Hilliard's (1994) monograph and consistent with our measurements, tube lengths of all other species sampled in clade 3 range from 17 to 60 mm. Some of this variation may be moving into any given population of *Z. microsiphon* through inter-species gene flow. Again using *Z. microsiphon*-S as an example, it is sister to a grade of populations of *Z. angustifolia*. The range of variation given for that species by Hilliard (1994) is 17-50 mm, which overlaps with that of *Z. microsiphon*-S. This gives two, non-mutually-exclusive hypotheses for the evolution of tube length variation in *Z. microsiphon*. One is that pollinator-mediated selection favoring particular mutations/recombinations is driving very rapid tube length evolution within populations. The other is that hybridization with night-flowering species has increased tube length variation in populations of *Z. microsiphon*, and this may have accelerated rates of pollinator-mediated floral evolution in this species. The correlation between tube and proboscis lengths found by Anderson

and Johnson (2008) is indicative of a role for coevolution between long-proboscid flies and *Z. microsiphon*. However, the phylogenetic data for *Zaluzianskya* presented here paint a more complex picture of possible convergent evolution of day-opening flowers pollinated by long-proboscid flies.

While the cpDNA data were more informative for clade 3 than ITS, clearly more characters are needed to attempt robust inference of relationships among these species and populations. Given the conflict seen between loci and at least some potential for hybridization, a comparison is also needed with other data sources. We have conducted ISSR analyses within this clade (Archibald J.K., Anderson B., Johnson S.D., unpub.) and found that even such hypervariable molecular markers struggle in resolving these relationships. A phylogenomic approach may be required to provide an evolutionary framework for this clade (Andrews et al., 2016; Mort et al., 2015; Wessinger et al., 2016).

5. Conclusions and future directions

This work demonstrates the lack of monophyly of *Zaluzianskya* and *Phyllopodium* and the need for a phylogeny across the latter. The inclusion of *Reyemia* and some species of *Phyllopodium* expands the known morphological diversity within the *Zaluzianskya* clade, particularly in floral and inflorescence characters. Such traits were already known to be variable in *Zaluzianskya*, suggesting potential roles for pollinator interactions in the evolution of these species (Johnson, 2006). Such a role is supported by study thus far of the interactions between *Z. microsiphon* and its pollinating long-proboscid fly (Anderson and Johnson, 2008), although the role of hybridization requires further study. These results establish the placement of disjuncts within *Zaluzianskya* and

the broad phylogenetic division of *Z. microsiphon*, but many questions remain. This is particularly notable within clade 3, with many relationships being unresolved. In addition to *Z. microsiphon*, other species complexes have been identified by this and previous work (Archibald et al., 2005), including the *Z. villosa* complex (*Z. villosa*, *Z. affinis*, and *Z. gracilis*) and the potential progenitor-derivative pair of *Z. pusilla* and *Z. divaricata*. We sampled additional populations from each, further substantiating the complicated nature of their evolution. Continued population sampling and more data are needed to better elucidate the patterns and processes of evolution within the *Zaluzianskya* clade.

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Fig. 1. Photographs illustrating a sample of the morphological diversity of *Zaluzianskya*. (A) *Z. pulvinata*, sect. *Nycterinia*. (B) *Z. microsiphon*, sect. *Nycterinia*. (C) *Z. pusilla*, sect. *Holomeria*. (D) *Z. affinis*, sect. *Zaluzianskya* subsect. *Zaluzianskya*.

Fig. 2. Phylogeny across major clades of tribe Limoselleae, with focus on the *Zaluzianskya* clade, as inferred from level 3 (see Table 2) Bayesian and maximum parsimony concatenated analyses. Node support for concatenated, ITS, and *trnL-trnF* analyses is given in that order, with jackknife values (JK) above the branch and posterior probabilities (PP) below. * = JK>70, PP>0.95, - = node with lower support or unresolved by that analysis, x = analysis strongly supports an alternate resolution (also indicated by black dashed branches), ^ = clade not relevant to the analysis due to unsampled accessions for that DNA region. The latter are also indicated by gray dashed branches and the DNA region available is noted after the accession name. Population ID is given to the right of taxon names, except for species with only one sampled population. Bars to the right of taxon names indicate major groups. *Phyllopodium* is in blue, former *Reyemia* in green, and *Zaluzianskya* in yellow.

Fig. 3. Phylogeny of the *Zaluzianskya* clade as inferred from level 1 (see Table 2) Bayesian and maximum parsimony concatenated analyses. Node support for concatenated, ITS, and cpDNA (*trnL-trnF*, *rpl16*, *rps16*, and *trnS-trnfM*) analyses is given in that order, with jackknife values (JK) above the branch and posterior

probabilities (PP) below. * = JK>70, PP>0.95, - = node with lower support or unresolved by that analysis, x = analysis strongly supports an alternate resolution (also indicated by black dashed branches). Gray dashed branches show the placement of individuals of *Phyllopodium* that were not included in level 1 analyses, but were sampled for levels 2 and/or 3. Population ID is given to the right of taxon names, except for species with only one sampled population. Bars to the right of taxon names note sectional affiliation (N: *Nycterinia*, M: *Macrocalyx*, Z: *Zaluzianskya*, R: *Reyemia*, H: *Holomeria*). *Phyllopodium* is in blue, former *Reyemia* in green, and *Z. microsiphon* in yellow.

Table 1. Taxonomic divisions within *Zaluzianskya* and *Phyllopodium*, sampling within each subgroup, and phylogenetic placement of sampled species of *Phyllopodium* (i.e., with the outgroup or with the *Zaluzianskya* clade).

Genus	Infrageneric groups			No. sampled species	No. species
<i>Zaluzianskya</i>	Section <i>Holomeria</i>			3	10
	Section <i>Macrocalyx</i>			1	5
	Section <i>Nycterinia</i>			14	20
	Section <i>Reyemia</i>			2	2
	Section <i>Zaluzianskya</i>	subsect. <i>Noctiflora</i>		2	4
		subsect. <i>Zaluzianskya</i>		7	17
<i>Phyllopodium</i>	Subgenus <i>Leiospermum</i>			1 - <i>Zal</i>	4
	Subgenus <i>Phyllopodium</i>	Group 1	Subgroup 1A	2 - OG	4
			Subgroup 1B	2 - OG	3
			Subgroup 1C	0	5
		Group 2	Subgroup 2A	2 - <i>Zal</i>	3
			Subgroup 2B	0	5
			"Anomalous species"	0	2

Table 2. Characteristics of final concatenated DNA sequence datasets and resulting maximum parsimony (MP) and Bayesian inference (BI) analyses of the *Zaluzianskya* clade (*Zal*) and outgroups (OG). The *Zal* clade includes former members of *Reyemia* (now *Zaluzianskya* sect. *Reyemia*) and several of the sampled species of *Phyllopodium*. Ranges for numbers of accessions reflect minor differences in sampling between concatenated, ITS, and cpDNA analyses.

	Level 1: Focused analysis of <i>Zal</i>	Level 2: <i>Zal</i> with broader OG sampling but more missing data	Level 3: <i>Zal</i> and representatives across Tribe Limoselleae with fewer loci
Data	ITS, <i>trnL-F</i> , <i>rpl16</i> , <i>rps16</i> , <i>trnS-fM</i>	ITS, <i>trnL-F</i> , <i>rpl16</i> , <i>rps16</i> , <i>trnS-fM</i>	ITS, <i>trnL-F</i>
No. pops. (<i>Zal</i> , OG)	81 (77, 4)	90-91 (78, 12-13)	125-130 (80-83, 45-50)
No. species (<i>Zal</i> , OG)	34 (30, 4)	43-44 (31, 12-13)	76-80 (32, 44-48)
No. genera	4	9-10	22-23
No. characters (nuc., indel)	4934 (4807, 127)	4934 (4807, 127)	1759 (nuc. only)
No. potentially parsimony informative chars.	508 (423 in <i>Zal</i>)	764 (427 in <i>Zal</i>)	476 (163 in <i>Zal</i>)
MP analyses			
No., length of most parsimonious trees	24, 1084	108, 1694	2533, 1451
CI	0.668	0.667	0.489
RI	0.917	0.897	0.856
RC	0.612	0.598	0.418
BI analyses			
Best-fit model	TIM1+G	TIM1+I+G	TIM1+I+G
No. generations	1,215,750,000	1,215,750,000	662,400,000

Appendix. Populations sampled for phylogenetic analyses of *Zaluzianskya* F.W. Schmidt and related genera. Geographic location and collection number are given. GenBank accession numbers for sequences of nuclear (ITS) and chloroplast (*trnL-trnF*, *rpl16*, *rps16*, and *trnS-trnfM*) DNA regions are listed in that order. [NOTE: GenBank numbers for new sequences will be added later].

Section/Subgroup ^a	Species	Pop. no. ^b	Location	Voucher (Herbarium); Indiv. sequenced	GenBank nos. (ITS, <i>trnL-trnF</i> , <i>rpl16</i> , <i>rps16</i> , <i>trnS-trnfM</i>) ^b
<i>Holomeria</i>	<i>Z. benthamiana</i> Walp.	1	29.6651 S 17.9985 E	J.K. Archibald 12 (OS); 1	AY712572, AY712650, AY712611, XX , XX
<i>Holomeria</i>	<i>Z. benthamiana</i>	2	32.8833 S 19.7112 E	J.K. Archibald 72 (OS); 1	XX, XX, XX, XX, XX
<i>Holomeria</i>	<i>Z. benthamiana</i>	3	29.6847 S 17.9017 E	J.K. Archibald 10 (OS); 1	XX, XX, XX, XX, XX
<i>Holomeria</i>	<i>Z. divaricata</i> (Thunb.) Walp.	1	34.0553 S 19.1652 E	J.K. Archibald 31 (OS); a	AY712569, AY712647, AY712608, XX , XX
<i>Holomeria</i>	<i>Z. divaricata</i>	2	34.0553 S 20.4475 E	J.K. Archibald & M.E. Mort 200 (KANU) ; 1	XX, XX, XX, XX, XX
<i>Holomeria</i>	<i>Z. pusilla</i> (Benth.) Walp.	1	32.8805 S 19.6640 E	J.K. Archibald 76 (OS); 2	AY712570, AY712648, AY712609, XX , XX
<i>Holomeria</i>	<i>Z. pusilla</i>	2	31.3798 S 19.0940 E	J.K. Archibald 26.1 (OS); 1	AY712571, AY712649, AY712610, XX , XX
<i>Macrocalyx</i>	<i>Z. mirabilis</i> Hilliard	1	32.5504 S 20.2626 E	J.K. Archibald 68 (OS); 1	AY712559, AY712637, AY712598, XX , XX
<i>Macrocalyx</i>	<i>Z. mirabilis</i>	2	32.2058 S 20.1771 E	A.D. Wolfe 956 (OS); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. angustifolia</i> Hilliard & B.L. Burtt	1	31.0307 S 30.1735 E	J.K. Archibald 119 (OS); 1	AY712550, AY712628, AY712589, XX , XX
<i>Nycterinia</i>	<i>Z. angustifolia</i>	2	ca. 32.6 S 27.0 E	C. Peter 9xi00 s.n. (NU); 1	AY712551, AY712629, AY712590, XX , XX
<i>Nycterinia</i>	<i>Z. capensis</i> (L.) Walp.	1	34.8205 S 20.0126 E	J.K. Archibald 2 (OS); 1	AY712555, AY712633, AY712594, XX , XX
<i>Nycterinia</i>	<i>Z. capensis</i>	2	34.4758 S 20.5092 E	J.K. Archibald 5 (OS); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. capensis</i>	3	34.1040 S 18.4310 E	J.K. Archibald & A.D. Wolfe 9 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. elongata</i> Hilliard & B.L. Burtt	1	30.7270 S 30.2969 E	J.K. Archibald & A.D. Wolfe 8 (OS); a	AY712552, AY712630, AY712591, XX , XX
<i>Nycterinia</i>	<i>Z. elongata</i>	2	25.2361 S	M.E. Mort, C.P. Randle, &	XX, XX, XX, XX, XX

			30.4892 E	J.K. Archibald 05.004 (KANU); 1	
<i>Nycterinia</i>	<i>Z. elongata</i>	3	29.4629 S 29.9628 E	J.K. Archibald 80 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. glareosa</i> Hilliard & B.L. Burt	1	29.7355 S 29.1880 E	J.K. Archibald 90 (OS); a	AY712556, AY712634, AY712595, XX, XX
<i>Nycterinia</i>	<i>Z. glareosa</i>	2	28.7458 S 28.8867 E	J.K. Archibald 104 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. katharinae</i> Hiern	1	Kirstenbosch Garden	A.D. Wolfe 729 (OS); 1	AY712557, AY712635, AY712596, XX, XX
<i>Nycterinia</i>	<i>Z. maritima</i> (L.f.) Walp.	1	34.0464 S 22.8417 E	J.K. Archibald 8 (OS); 1	AY712554, AY712632, AY712593, XX, XX
<i>Nycterinia</i>	<i>Z. maritima</i>	2	33.7449 S 26.5598 E	J.K. Archibald & M.E. Mort 232 (KANU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i> (O. Kuntze) K. Schum.	1	28.6860 S 28.9001 E	S.D. Johnson 19ii00 s.n. (NU); 1	AY712545, AY712623, AY712584, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	2	29.5976 S 29.3119 E	J.K. Archibald 98 (OS); a	AY712546, AY712624, AY712585, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	3	ca. 30.7 S 28.1 E	S.D. Johnson 30xii99 s.n. (NU); 1	AY712547, AY712625, AY712586, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	4	28.9944 S 29.2272 E	M.E. Mort, C.P. Randle, & J.K. Archibald 05.008; 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	5	29.8402 S 29.2142 E	J.K. Archibald 84 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	6	29.7417 S 29.1984 E	J.K. Archibald 89 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A2	28.6856 S 28.8981 E	B. Anderson 14 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A3	28.7417 S 28.8917 E	B. Anderson 16 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A4	29.2717 S 29.2715 E	B. Anderson 20 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A6	29.5889 S 29.3083 E	B. Anderson 25 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A7	29.6667 S 28.3667 E	B. Anderson s.n. (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A8a	29.8687 S	B. Anderson s.n. (STEU); 4	XX, XX, XX, XX, XX

<i>Nycterinia</i>	<i>Z. microsiphon</i>	A8b	29.1161 E 29.8687 S	B. Anderson s.n. (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A9	29.1161 E 29.8747 S	B. Anderson 17 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A10	29.7225 E 30.0525 S	B. Anderson 19 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A11	28.9306 E 30.1361 S	B. Anderson 24 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A12	28.6850 E 30.4028 S	B. Anderson 15 (STEU); 4	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A13	28.8189 E 30.6639 S	B. Anderson 22 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A14	29.5708 E 30.7292 S	B. Anderson 26 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A15	28.1397 E 30.7584 S	B. Anderson 23 (STEU); 11	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. microsiphon</i>	A16	28.2100 E 31.1833 S	B. Anderson 18 (STEU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. natalensis</i> Bernh. ex Krause	1	27.5849 E 29.8179 S	J.K. Archibald 114 (OS); a	AY712548, AY712626, AY712587, XX, XX
<i>Nycterinia</i>	<i>Z. ovata</i> (Benth.) Walp.	1	30.8026 E 29.4079 S	J. K. Archibald 111 (OS); a	AY712558, AY712636, AY712597, XX, XX
<i>Nycterinia</i>	<i>Z. ovata</i>	2	29.8918 E 29.2833 S	J. Archibald 99 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. pachyrrhiza</i> Hilliard & B.L. Burtt	1	30.2833 E ca. 27 S	S.D. Johnson 01 s.n. (NU); 1	AY712553, AY712631, AY712592, XX, XX
<i>Nycterinia</i>	<i>Z. pulvinata</i> Killick	1	32 E 29.2506 S	J.K. Archibald & A.D. Wolfe 5 (OS); a	AY712549, AY712627, AY712588, XX, XX
<i>Nycterinia</i>	<i>Z. pulvinata</i>	2	30.3182 E 29.6004 S	J.K. Archibald & A.D. Wolfe 4 (OS); a	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. schmitziae</i> Hilliard & B.L. Burtt	1	29.3040 E 25.6839 S	P. Burgoyne 24ii2005 s.n.; 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. spathacea</i> (Benth.) Walp.	1	28.0572 E 24.9381 S	M.E. Mort, C.P. Randle, & J.K. Archibald 05.071 (KANU); 1	XX, XX, XX, XX, XX
<i>Nycterinia</i>	<i>Z. tropicalis</i> Hilliard	1	30.8544 E ca. 18 S	N.C. Chase 8390 (K); MWC	XX, XX, -, XX, -

<i>Reyemia</i>	<i>Z. chasmanthiflora</i> (Hilliard) J.C. Manning & Goldblatt	1	32 E ca. 31 S 20 E	37673 A.U. Batten 1016 (K); MWC 37674	XX, XX, XX, –, XX
<i>Reyemia</i>	<i>Z. nemesioides</i> Diels	1	31.7274 S 19.7864 E	A.D. Wolfe 986 (OS); 1	AY712582, AY712660, AY712621, XX, XX
<i>Z. Noctiflora</i>	<i>Z. elgonensis</i> Hedberg	1	ca. 3 S 37 E	Friis 241 (K); MWC 37671	XX, XX, XX, XX, –
<i>Z. Noctiflora</i>	<i>Z. peduncularis</i> (Benth.) Walp.	1	32.9090 S 20.6095 E	J.K. Archibald 45 (OS); a	AY712568, AY712646, AY712607, XX, XX
<i>Z. Noctiflora</i>	<i>Z. peduncularis</i>	2	31.3549 S 19.8027 E	J.K. Archibald 23 (OS); 1	XX, XX, XX, XX, XX
<i>Z. Noctiflora</i>	<i>Z. peduncularis</i>	3	33.7299 S 23.0444 E	J.K. Archibald & M.E. Mort 242 (KANU); 2	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. affinis</i> Hilliard	1	31.6002 S 18.7549 E	J.K. Archibald 9 (OS); 1	AY712561, AY712639, AY712600, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. affinis</i>	2	31.5958 S 18.4389 E	J.K. Archibald 29 (OS); 1	AY712562, AY712640, AY712601, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. affinis</i>	3	29.1210 S 16.8468 E	J.K. Archibald 14 (OS); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. affinis</i>	4	30.5828 S 17.5403 E	K.E. Steiner 3358 (NBG); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. bella</i> Hilliard	1	32.9090 S 20.6095 E	J.K. Archibald 44 (OS); a	AY712564, AY712642, AY712603, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. bella</i>	2	32.8732 S 20.6712 E	J.K. Archibald 50 (OS); a	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. cohabitans</i> Hilliard	1	32.9090 S 20.6095 E	J.K. Archibald 46 (OS); 1	AY712567, AY712645, AY712606, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. cohabitans</i>	2	32.3006 S 20.3880 E	J.K. Archibald 60 (OS); 2	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. gracilis</i> Hilliard	1	34.4758 S 20.5095 E	J.K. Archibald 6.2 (OS); a	AY712563, AY712641, AY712602, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. gracilis</i>	2	34.7111 S 20.1036 E	J.K. Archibald & M.E. Mort 192 (KANU); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. gracilis</i>	3	34.3208 S 21.5034 E	J.K. Archibald & M.E. Mort 263 (KANU); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. minima</i> (Hiern) Hilliard	1	32.2084 S	J.K. Archibald 58 (OS); a	AY712566, AY712644, AY712605, XX,

<i>Z. Zaluzianskya</i>	<i>Z. minima</i>	2	20.4257 E 31.7268 S 20.0495 E	K.E. Steiner 3396 (NBG); 1	XX XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. villosa</i> F.W. Schmidt	1	34.5647 S 19.3528 E	J.K. Archibald 1 (OS); 1	AY712560, AY712638, AY712599, XX , XX
<i>Z. Zaluzianskya</i>	<i>Z. villosa</i>	2	34.3123 S 18.4508 E	J.K. Archibald & M.E. Mort 155 (KANU); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. violacea</i> Schl.	1	31.6359 S 19.8963 E	J.K. Archibald 18 (OS); 1	AY712565, AY712643, AY712604, XX , XX
<i>Z. Zaluzianskya</i>	<i>Z. violacea</i>	2	31.4290 S 19.7844 E	J.K. Archibald 17.1 (OS); 1	XX, XX, XX, XX, XX
<i>Z. Zaluzianskya</i>	<i>Z. violacea</i>	3	29.7589 S 17.9352 E	J.K. Archibald 15 (OS); 1	XX, XX, XX, XX, XX
Subgenus <i>Leiospermum</i>	<i>Phyllopodium</i> cf. <i>cephalophorum</i> (Thunb.) Hilliard	GB			AJ584751, AJ401447, –, –, –
Subgenus <i>Ph.</i> Subgroup 2A	<i>Phyllopodium cordatum</i> (Thunb.) Hilliard	1	34.5392 S 20.0344 E	J.K. Archibald & M.E. Mort 179 (KANU); 1	XX, XX, XX, XX, XX
Subgenus <i>Ph.</i> Subgroup 1A	<i>Phyllopodium cuneifolium</i> (L.f.) Benth.	1	ca. 33.5 S 27.1 E	J.K. Archibald & M.E. Mort 229 (KANU); 1	XX, XX, XX, XX, XX
Subgenus <i>Ph.</i> Subgroup 1A	<i>Phyllopodium cuneifolium</i>	GB			–, AJ296496, –, –, –
Subgenus <i>Ph.</i> Subgroup 1B	<i>Phyllopodium dolomiticum</i> Hilliard	GB			AJ584806, AJ586087, –, –, –
Subgenus <i>Ph.</i> Subgroup 2A	<i>Phyllopodium mimetes</i> Hilliard	1	34.0492 S 18.6688 E	A.D. Wolfe 936 (OS); 1	XX, XX , –, –, –
Subgenus <i>Ph.</i> Subgroup 1B	<i>Phyllopodium multifolium</i> Hiern	GB			AJ584802, AJ586085, –, –, –
Subgenus <i>Ph.</i> Subgroup 1A	<i>Phyllopodium rustii</i> (Rolfe) Hilliard	1	33.5147 S 22.3363 E	J.K. Archibald & M.E. Mort 248 (KANU); 1	XX, XX, XX, XX, XX
–	<i>Aptosimum indivisum</i> Burch. ex Benth.	1	33.1267 S 21.2054 E	A.D. Wolfe 910 (OS); 1	AY712574, AY712652, AY712613, –, –
–	<i>Aptosimum procumbens</i> (Lehm.) Steud.	1	Karoo Botanical Garden	A.D. Wolfe 685 (OS); 1	AY712573, AY712651, AY712612, –, –
–	<i>Barthlottia</i> <i>madagascariensis</i> Eb. Fisch.	GB			AJ550576, AJ401444, –, –, –

–	<i>Chaenostoma hispidum</i> (Thunb.) Benth.	1	34.1683 S 18.3441 E	A.D. Wolfe 930 (OS); 1	AY712583, AY712661, AY712622, –, –
–	<i>Chaenostoma patrioticum</i> (Hiern) Kornhall	GB			AJ550612, AJ551261, –, –, –
–	<i>Chenopodiopsis retrorsa</i> Hilliard	GB			AJ584757, AJ296502, –, –, –
–	<i>Cromidon decumbens</i> (Thunb.) Hilliard	GB			AJ584756, AJ296503, –, –, –
–	<i>Dischisma ciliatum</i> (Berg.) Choisy	GB			–, AJ296501, –, –, –
–	<i>Dischisma spicatum</i> Choisy	GB			AJ584823, AJ586098, –, –, –
–	<i>Glekia krebsiana</i> (Benth.) Hilliard	GB			–, AJ296519, –, –, –
–	<i>Glumicalyx flanaganii</i> (Hiern) Hilliard & B.L. Burt	1	ca. 29.5 S 29.2 E	T.J. Edwards 1774 (NU); 1	AY712575, AY712653, AY712614, –, –
–	<i>Glumicalyx goseloides</i> (Diels) Hilliard & B.L. Burt	1	Not available	K.E. Steiner 4ii98 s. n. (NBG); 1	AY712576, AY712654, AY712615, XX , XX
–	<i>Hebenstretia dura</i> Choisy	GB			AJ584821, AJ586097, –, –, –
–	<i>Hebenstretia lanceolata</i> Rolfe	GB			AJ584825, AJ586100, –, –, –
–	<i>Jamesbrittenia adpressa</i> (Dinter) Hilliard	1	ca. 29.5 S 19.4 E	D.A. Snijman 1851 (NBG); 1	AY712577, AY712655, AY712616, –, –
–	<i>Jamesbrittenia filicaulis</i> (Benth.) Hilliard	GB			–, AJ621112, –, –, –
–	<i>Jamesbrittenia megadenia</i> Hilliard	GB			AJ550584, AJ296511, –, –, –
–	<i>Limosella grandiflora</i> Benth.	GB			AJ550587, AJ550525, –, –, –
–	<i>Limosella macrantha</i> R.E. Fr.	GB			AJ550586, AJ550526, –, –, –
–	<i>Lyperia antirrhinoides</i> (L.f.) Hilliard	GB			AJ616324, AJ621123, –, –, –
–	<i>Lyperia tristis</i> (L.f.) Benth.	1	31.7267 S 18.6667 E	A.D. Wolfe 879 (OS); 1	AY712578, AY712656, AY712617, –, –
–	<i>Lyperia tristis</i>	GB			AJ550589, AJ550527, –, –, –
–	<i>Manulea altissima</i> L. f.	1	31.7267 S	A.D. Wolfe 708 (OS); 1	AY712579, AY712657, AY712618, –, –

			18.6667 E		
–	<i>Manulea calciphila</i> Hilliard	GB			AJ550592, AJ550533, –, –, –
–	<i>Manulea chrysantha</i> Hilliard	GB			AJ550595, AJ550532, –, –, –
–	<i>Manulea rubra</i> (Berg.) L. f.	1	32.6203 S 19.1170 E	A.D. Wolfe 718 (OS); 1	AY712580, AY712658, AY712619, –, –
–	<i>Melanospermum foliosum</i> (Benth.) Hilliard	GB			AJ584753, AJ296507, –, –, –
–	<i>Melanospermum transvaalense</i> (Hiern) Hilliard	GB			AJ584759, AJ296508, –, –, –
–	<i>Microdon dubius</i> (L.) Hilliard	GB			AJ584792, AJ586079, –, –, –
–	<i>Microdon orbicularis</i> Choisy	GB			AJ584762, AJ586053, –, –, –
–	<i>Polycarena aurea</i> Benth.	1	32.3260 S 20.1354 E	A.D. Wolfe 979 (OS); 1	AY712581, AY712659, AY712620, XX , XX
–	<i>Polycarena batteniana</i> Hilliard	GB			AJ584769, AJ586059, –, –, –
–	<i>Polycarena capensis</i> (L.) Benth.	GB			–, AJ586050, –, –, –
–	<i>Polycarena filiformis</i> Diels	GB			AJ584807, AJ586088, –, –, –
–	<i>Polycarena formosa</i> Hilliard	GB			AJ584754, AJ296515, –, –, –
–	<i>Polycarena pubescens</i> Benth.	GB			AJ584768, –, –, –, –
–	<i>Pseudoselago ascendens</i> (E. Mey.) Hilliard	GB			–, AJ296495, –, –, –
–	<i>Pseudoselago densifolia</i> (Hochst.) Hilliard	GB			AJ584799, AJ586082, –, –, –
–	<i>Pseudoselago recurvifolia</i> Hilliard	GB			AJ584801, AJ586084, –, –, –
–	<i>Selago canescens</i> L.f.	GB1			–, AJ608637, –, –, –
–	<i>Selago canescens</i>	GB2			AJ584772, –, –, –, –
–	<i>Selago foliosa</i> Rolfe	GB			AJ584786, AJ586073, –, –, –
–	<i>Selago venosa</i> Hilliard	GB			AJ584778, AJ586066, –, –, –
–	<i>Sutera foetida</i> Roth	GB			AJ550609, AJ296510, –, –, –
–	<i>Tetraselago longituba</i>	GB			AJ584826, AJ296506, –, –, –

	(Rolfe) Hilliard & B.L. Burt				
–	<i>Tetrasselago wilmsii</i> (Rolfe) Hilliard & B.L. Burt	GB			AJ584827, AJ586101, –, –, –
–	<i>Trieneea taylorii</i> Hilliard	1	ca. 32 S 19 E	Taylor 11401 (STE); 1	XX , –, –, –, –
–	<i>Trieneea glutinosa</i> (Schltr.) Hilliard	GB			AJ584758, AJ296516, –, –, –

^a Infrageneric membership is given for *Zaluzianskya* and *Phyllopodium*; the two subsections of section *Zaluzianskya* are indicated as *Z. Zaluzianskya* and *Z. Noctiflora*. All genera are members of tribe Limoselleae except *Aptosimum* (tribe Aptosimeae).

^b Bold indicates populations and sequences that have not been previously published in a phylogenetic analysis.

***Zaluzianskya* clade**