A Phylogenetic Analysis of Hybanthus: Reclassifying *Hybanthus concolor* (Green Violet)

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Introduction

The genus *Hybanthus* of the Violet Family (Violaceae) is non-monophyletic, meaning that not all species of *Hybanthus* share a most recent common ancestor. Previous phylogenetic analysis in the Violet Family suggested that *Hybanthus concolor* from eastern North America was resolved as a sister taxon to a clade of two *Hybanthus* species from the Caribbean region (Wahlert et al., 2014). The two clades display distinct differences in morphology, climate, and geographic distribution. **In this project, we conducted expanded DNA sequencing of North American and Caribbean *Hybanthus* species to test the hypothesis that these two groups represent distinct evolutionary genera.**

Fig. 1 *H. concolor* (Missouri Botanical Garden)

Fig. 2 *H. concolor* (Center for Plant Conservation)

Methodology

Taxon Sampling:

- Added 3 accessions of *Hybanthus concolor*, 4 *Hybanthus havanensis*, and 3 *Hybanthus yucatanensis* to the Wahlert et al. (2014) matrix (115 Violaceae).

Extraction, Amplification, & Sequencing:

- DNA was extracted from herbarium specimens with the DNEasy Plant Mini Kit
- Amplified the *trnL* (UAA) intron and *trnL* (UAA)–*trnF* (GAA) intergenic spacer with primer pairs Tab C + Tab F (one fragment) or Tab C + Tab D and Tab E + Tab F (two fragments).
- Amplified *rbcL* Rubisco gene region in two fragments, *rbcL I* and *rbcL II*, with primer pairs rbcL F + rbcL 724R and rbcL 536F + rbcL 3R, respectively.
- PCR reactions: 25 ml reactions, 10–20 ng DNA, 1.0 unit Phusion™ Plus DNA Polymerase, 2.5 ml 10 +buffer with $MgCl_2$, 1.0 ml dNTPs, and 0.63 ml 20 mmol primers. Followed an initial denaturation (94°C, 2 min), 40 cycles of denaturation (94°C, 30 sec), annealing (54-57°C, 1 min), and elongation (72°C, 1 min), final extension (72°C, 7 min).
- Annealing temperatures: 54°C (*trnL/trnL-F*), 57°C (*rbcL I*), and 54°C (*rbcL II*).
- PCR products were examined by electrophoresis at 100 V in a 1.3% agarose gel stained with SYBR Safe DNA Gel Stain and then cleaned by DNeasy PowerClean Pro Cleanup Kit.
- Sequencing was carried out at the University of Arizona with the same primers and annealing temperatures as the PCR reaction.

Phylogenetics:

- Sequences were inspected and aligned manually using BioEdit (Hall 1999).
- Maximum parsimony (MP) analyses were conducted in PAUP* v4.0b10 (Swofford 2002) using a heuristic search strategy with TBR branch swapping, 1,000 random addition replicates, saving one tree per replicate, steepest descent off, and MULTREES in effect.
- All characters were equally weighted and unordered. Internal branch support of phylogenetic trees from each MP analysis was estimated with 10 bootstrap (BS) replicates (Felsenstein 1985) using a full heuristic search with TBR branch swapping, 10 random stepwise addition replicates, and MULTREES in effect.

	trnL/trnL-F	*rbcL*	*trnL/trnL-F + rbcL*
Aligned length:	999	1296	2295
Parsimony informative sites:	395 (39.5%)	265 (20.4%)	660 (28.9%)
Constant sites:	833 (59.7%)	870 (67.1%)	1703 (74.2%)
Number of MPTs:	> 27,000	> 100,000	> 20,000

Fig. 3 Maximum parsimony cladogram statistics

Results

The pattern of phylogenetic relationships supported what was found in Wahlert et al. (2014). The four accessions of *Hybanthus concolor* were resolved in a clade with parsimony BS of 100 and were recovered as sister to the clade containing eight accessions of *H. havanensis* and *H. yucatanensis* (BS = 100).

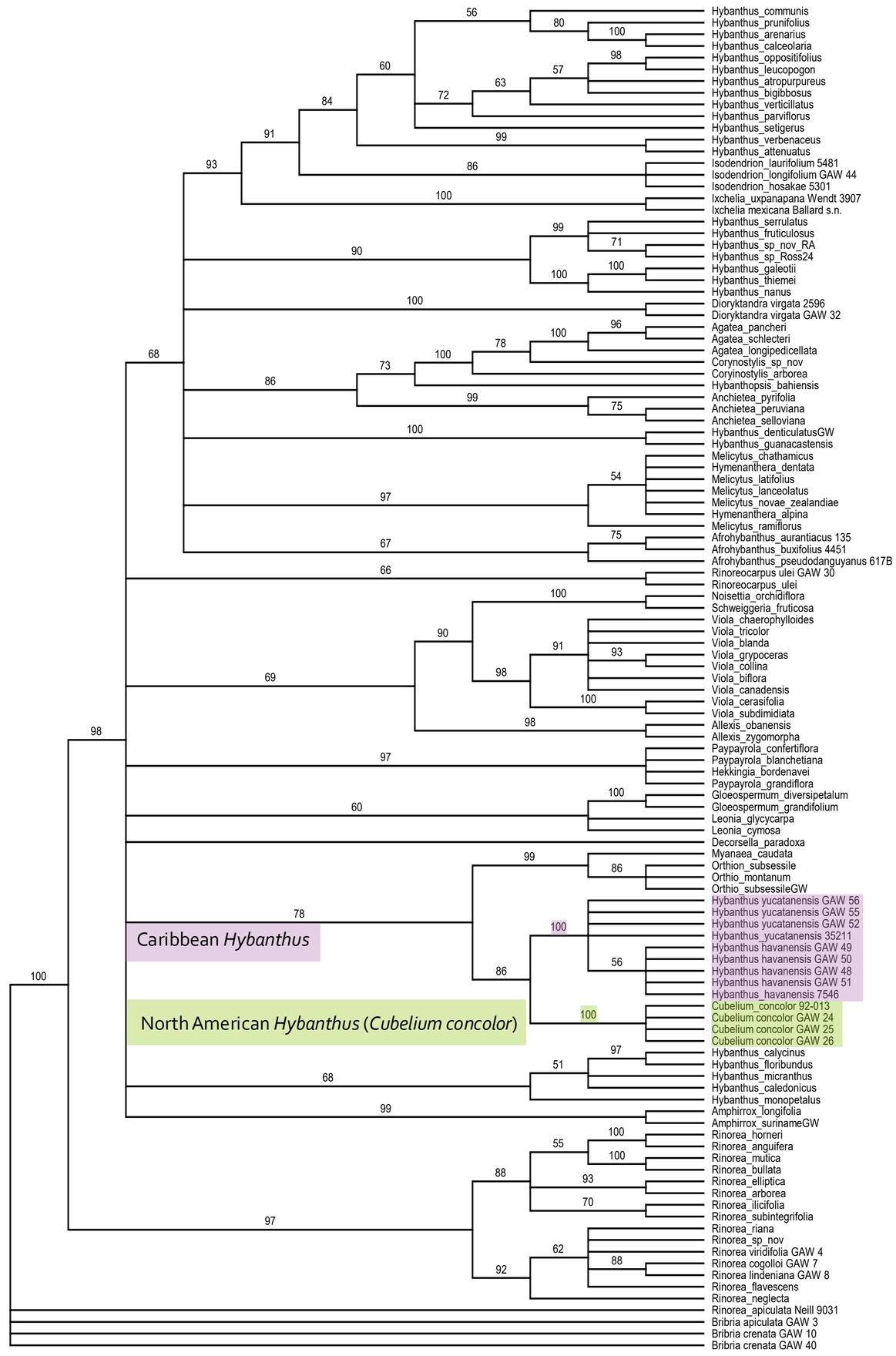

Fig. 4 Maximum parsimony cladogram (bootstrap values above the branches)

Conclusion

The results for our Caribbean and North American *Hybanthus* clades were consistent with those of Wahlert et al. (2014). Our expanded taxon sampling of *Hybanthus concolor*, *Hybanthus havanensis*, and *Hybanthus yucatanensis* strengthens the findings of Wahlert et al. (2014), and combined with additional morphological evidence, supports recognizing these two groups as separate genera. Therefore, we propose transferring *Hybanthus concolor* to an earlier available generic name, *Cubelium*. Correct taxonomic classification is crucial for analyzing evolutionary history and relationships, accurate scientific communication, and can be applicable to conservation efforts.

Acknowledgements

This work was generously supported by the UC Santa Barbara Undergraduate Research & Creative Activities URCA grant. Thank you to my research mentor, Dr. Greg Wahlert, for his expert guidance and support throughout this project.

Wahlert, G. A., Marcussen, T., de Paula-Souza, J., Feng, M., & Ballard, H. E. (2014). A Phylogeny of the Violaceae (Malpighiales) Inferred from Plastid DNA Sequences: Implications for Generic Diversity and Intrafamilial Classification. *Systematic Botany*, *39*(1), 239–252. http://www.jstor.org/stable/24546134

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