Identification and characterization of *Gypsophila paniculata* color morphs in Sleeping Bear Dunes National Lakeshore, MI, USA.

Marisa L. Yang[1] , Emma Rice[2], Hailee Leimbach-Maus[2], and Charlyn G. Partridge[2]

[1]Environmental Science Policy, and Management, University of California, Berkeley, 260 Mulford Hall, Berkeley, CA 94720, email: marisayang79@gmail.com
[2]Annis Water Resources Institute, 740 W. Shoreline Dr., Muskegon, MI 49441, email: riceemm@mail.gvsu.edu; haileepavischi@gmail.com

Corresponding author: Charlyn Partridge, Annis Water Resources Institute, Grand Valley State University, 740 W. Shoreline Dr., Muskegon, MI; 49441, email: partridc@gvsu.edu

Abstract

Gypsophila paniculata (baby's breath) is an invasive species found throughout much of the northwest United States and western Canada. Two distinct color morphs of baby's breath were identified in areas within the coastal dunes along eastern Lake Michigan. The more common morph produces stems that are purple in color (purple morph), while the atypical morph has stems that are green-yellow (green-yellow morph). The purpose of this study was to characterize these morphs and determine if they are genetically distinct species in order to assess whether alternative management strategies should be employed to control these populations. We sequenced two chloroplast regions, RbcL and MatK, and one nuclear region, ITS2, from the purple morphs and green-yellow morphs collected from Sleeping Bear Dunes National Lakeshore, MI, USA. Sequences were aligned with reference *G. paniculata* and *G. elegans* sequences obtained from GenBank and the Barcode of Life (BOLD) databases. Phylogenetic analyses suggest that the two color morphs belong *to G. paniculata* and do not appear to be distinct genetic strains. We propose that current management practices continue to treat the two color morphs in a similar manner in terms of removal to prevent the further spread of this species.

Introduction

The Great Lakes sand dunes comprise the most extensive freshwater dune complex in the world, stretching over 1,000 km^2 in Michigan alone. Within northwest Michigan, the sand dunes ecosystem is vital both environmentally and economically. It is home to a number of threatened and endangered species, including piping plover (*Charadrius melodus*) and Pitcher's thistle (*Cirsium pitcheri).* The colonization of invasive species into this region can significantly alter the biological composition of native communities (Olson 1958, Leege and Murphy 2001, Emery et al. 2013). One invasive species of significant concern is the perennial baby's breath (*Gypsophila paniculata*). In 2015, baby's breath was listed by the Department of Natural Resources (DNR) as a "priority" invasive species for detection and control in Michigan's northern lower peninsula (DNR 2015). Since its colonization in the region it has spread along a 260 km stretch of the Michigan shoreline. Baby's breath produces a large primary taproot that can extend down to 4 meters in depth, which likely helps it outcompete native vegetation for limited resources (Darwent and Coupland 1966, Karamanski 2000). The presence of baby's breath in this system is associated with a reduction in the number of threatened Pitcher's thistle and an increase in the number of other non-native species (Emery et al. 2013). In addition, while many of the vulnerable and endangered plant species in these areas are seed limited, (e.g., Pitcher's thistle produces approximately 50-300 seeds per plant (Bevill et al. 1999)), baby's breath can produce up to 14,000 seeds per plant annually (Stevens 1957), effectively outcompeting native species in terms of overall yield. This has led to baby's breath composing approximately 50-80% of the ground cover in some areas (Karamanski 2000, Emery et al. 2013).

One concern with current management efforts is that there is some anecdotal evidence that there may be a new baby's breath variant (morph) within the Michigan dune system. In 2011 and 2012 The Nature Conservancy (TNC) removal crews reported baby's breath plants with different character traits than what is commonly observed (TNC 2014). The atypical morph has stems and leaves that are lighter in color and more yellow than the common purple morph (Figure 1a-c). The purple morph has a thick taproot (4-7 cm in diameter) just below the caudex that remains unbranched for approximately 60 – 100 cm (Darwent & Coupland 1966). Severing just below the intersection of the caudex and the taproot is where manual removal efforts target to limit regrowth. However, the atypical green-yellow morph's root system was reported by TNC to be more diffuse, making it harder to identify a primary taproot and thus, harder to sever without the potential for regrowth (TNC 2014). Currently, these green-yellow morphs are treated with herbicide application (glyphosate) when observed; however, if this morph continues to spread into areas where threatened or endangered species are present, removal methods will be a primary concern. If the green-yellow morph and the purple morph represent two discrete species, then alternative management strategies may need to be considered for these populations.

One of the first steps toward adapting current management strategies for this population is to identify whether the green-yellow morph is genetically distinct from the purple morph. While *G. paniculata* is the dominant invasive baby's breath species in northwest Michigan, there are a number of distinct *G. paniculata* commercial strains that could potentially invade these areas considering seeds are commonly sold in stores throughout the region (personal observation). In addition, other baby's breath species, including *G. elegans* are sold in this region in commercial wildflower packets. *G. elegans* is an annual species that has a smaller taproot compared to *G. paniculata,* and its coloration is similar to that of the green-yellow morph. Thus,

79 the goal of this work was to use traditional 'barcode' genes for plants to characterize the genetic
80 relationship between the green-yellow morph and the common purple morph.
81
82 **Methods and materials**
83
84 *DNA Extraction, Amplification, and Sequencing*
85
86 We collected leaf tissue from 1 green-yellow morph and 16 purple morphs in 2016 and
87 an additional 15 green-yellow morphs in 2017 from Sleeping Bear Dunes National Lakeshore
88 (SBDNL), Empire, MI, USA (specifically: 44.884941 N, 86.062111 W and 44.875302 N,
89 86.056821 W). Leaf tissue was dried in silica gel until DNA extractions could take place. DNA
90 was extracted using Qiagen DNeasy Plant Mini Kits (Qiagen, Hilden, Germany). After
91 extraction, the DNA samples were placed through Zymo OneStep PCR inhibitor removal
92 columns (Zymo, Irvine, CA) to remove any secondary metabolites that might inhibit PCR
93 amplification. The DNA for each sample was then quantified using a NanoDrop 2000
94 (ThermoFisher, Waltham, MA).

95 The DNA of both green-yellow morphs and purple morphs was amplified at three
96 proposed 'barcoding' genes for plants: large subunit of the ribulose-bisphosphate carboxylase
97 gene (RbcL), maturase K (MatK), and internal transcribed spacer 2 (ITS2). The RbcL region was
98 amplified using RbcL 1F and RbcL 724R primers (Chen et al. 2010), MatK was amplified using
99 MatK 390F and MatK 1440R primers (Fior et al. 2006), and the ITS2 region was amplified using
100 ITS2 2SF and ITS2 S3R primers (Chen et al. 2010). Individual PCR reactions for all loci
101 consisted of 1X Taq Buffer, 2.0 mM $MgCl_2$, 0.3 μM dNTP, 0.08 mg/mL BSA, 0.4 μM forward
102 and reverse primers, and 0.5 units of Taq polymerase in a 20 μL reaction volume. The thermal
103 cycle protocols consisted of the following: for RbcL, an initial denaturing step of 95ºC for 2

minutes, followed by 35 cycles of 94ºC for 1 minute, 55ºC for 30 seconds, and 72ºC for 1 minute. A final elongation step was performed at 72ºC for 7 minutes. For MatK, the thermal profile consisted of 26 cycles of 94ºC for 1 minute, 48ºC for 30 seconds, and 72ºC for 1 minute, followed by a final elongation step at 72ºC for 7 minutes. For ITS2, an initial denaturing step of 95ºC for 2 minutes was applied, followed by 35 cycles of 95ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 1.5 minutes, and a final elongation step of 72ºC for 8 minutes. Successful amplification was checked by running the PCR product on a 2% agarose gel stained with ethidium bromide. PCR reactions were then cleaned using ExoSAP-IT PCR Product Cleanup Reagent (ThermoFisher, Waltham, MA). Sequencing reactions were performed for both forward and reverse primers for each of the three genes. Sequencing reactions were cleaned using a Sephadex column (GE Healthcare Life Science, Marlborough, MA) and sequenced on an ABI Genetic BioAnalyzer 3130xl (Applied Biosystems, Foster City, CA). Out of the 16 green-yellow morphs a total of 13 were successfully sequenced for RbcL, 13 were successfully sequenced for MatK, and 14 were successfully sequenced for ITS2. For the purple morphs a total of 15, 12, and 15 individuals were successfully sequenced for RbcL, MatK and ITS2, respectively.

G. paniculata and *G. elegans* reference sequences for RbcL, MatK, and ITS2 were downloaded either from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) or the Barcode of Life Database (BOLD) (http://www.barcodeoflife.org). Their accession numbers and sequences are provided in Supplemental Table 1. All fasta files corresponding to these data will be deposited in the Dryad database and sequences will be submitted to GenBank.

Alignment and Phylogenetic Analysis

All success sequences from our field samples, as well as sequences for *G. elegans* and *G. paniculata* obtained from GenBank or BOLD, were imported into the program

127 MEGA7 (version 7.0.14) (Kumar et al. 2016) and sequences for each of the three genes
128 were aligned both individually and with all sequences combined using Muscle (Edgar
129 2004). The total number of base pairs aligned and analyzed for each region included: 516
130 base pair (bp) for RbcL, 720 bp for MatK, 264 bp for ITS2, and 1498 bp for the three regions
131 combined. All alignment parameters were kept at their default settings. Once aligned, we used
132 MEGA7 to identify the most appropriate substitution model (RbcL: Jukes-Cantor, MatK:
133 Tamura 3-parameter, ITS2: Jukes-Cantor, all genes combined: Tamura 3-parameter). We then
134 created phylogenetic trees using a maximum-likelihood (ML) approach with 500-replicated
135 bootstrap analyzes, as well as using neighbor joining, and parsimony models.

136

137 **Results and Discussion**

138 Our results indicate that the green-yellow morph identified in SBDNL is not a genetically
139 distinct species from the common purple *G. paniculata* found throughout SBDNL. The RbcL,
140 MatK, ITS2, and combined dataset showed similar patterns with both the green-yellow morphs
141 and the purple morphs clustering together with a *G. paniculata* reference sequence, and
142 separately from *G. elegans* reference sequences (Figure 2a-d; Supplemental Figure 1). This
143 suggests that both color morphs belong to *G. paniculata*.

144 Of the regions analyzed for the green-yellow morphs, purple morphs, and reference
145 sequences, RbcL was the most conserved sequence with an overall mean genetic distance (*d)* =
146 0.001, followed by MatK (d = 0.002) and ITS2 (d = 0.018). For the ITS2 region, there were 6
147 purple *G. paniculata* morphs and one green-yellow morph that clustered together inside the *G.*
148 *paniculata* branch (Figure 2a & 2d). Further examination of the chromatograms for these

individuals show that they are likely heterozygous at position 195 of our aligned sequence and amplification bias of the 'A' SNP over the allele containing the 'G' SNP is driving this pattern.

The phylogeny constructed from the RbcL sequence showed one *G. paniculata* reference sequence (SDH831-14) grouping together with *G. elegans*. All reference sequences for RbcL were obtained from the BOLD database, and it is possible that this specific individual was misidentified. Further examination of this specimen from the herbarium image posted to BOLD indicates a small taproot compared to the upper foliage (http://www.boldsystems.org/pics/SDH/CCDB-24938-B09_h235508%2B1424896806.jpg), which is one characteristic of the annual *G. elegans*. While there is also sequence data for both the ITS2 and MatK regions for this specimen in BOLD, these were not used for this dataset due to higher levels of sequence fragmentation compared to our samples. However, both the ITS2 and MatK sequences from this *G. paniculata* reference sequence aligned most closely to *G. elegans* in BLAST.

While our data suggest that both the purple and green-yellow morphs are *G. paniculata*, whether the green-yellow morph is a different commercial genetic strain is still unclear. RbcL, MatK, and ITS2 are common 'barcode' genes used to delineate plant species, but their ability to distinguish genetically distinct strains of the same species can be limited. RbcL and MatK are highly conserved and would likely not distinguish between different cultivars; however, ITS2 is more variable and has been used to distinguish between different varieties of domestic and imported teas (*Camellia sinensis*) (Lee et al. 2017). In our dataset, the purple and green-yellow morphs from SBDNL grouped separately from the *G. paniculata* reference sequence, but within the same clade. The *G. paniculata* reference sequence for ITS2 was obtained from GenBank and is part of an unpublished study, so we do not have information regarding where this sample was

172 collected. However, given the distinct grouping between the *G. paniculata* reference sequence
173 and our samples, ITS2 may be a promising region to assess the ability to distinguish among
174 different commercial strains or regional varieties of *G. paniculata*. If this is the case, then our
175 data would suggest that the purple and yellow-green morphs not only belong to the same species
176 (*G. paniculata)*, but are likely derived from the same or similar initial source populations.

177 The mechanism driving the color difference between the purple and green-morphs is
178 currently unknown. Within SBDNL, the purple morph is the most common form, with green-
179 yellow individuals found interspersed in a couple of locations throughout the dunes. The largest
180 observed group of green-yellow morphs consists of a few hundred plants clumped within
181 approximately an acre-sized area and interspersed throughout large groups of purple morphs.
182 Occasionally, small pockets of green-yellow individuals have also been found together in other
183 areas of SBDNL and Zetterberg Preserve (approximately 38 km from SBDNL). Based upon the
184 dispersal patterns of the two morphs throughout the dunes, the color difference observed does
185 not appear to be solely environmentally driven, and likely has a genetic component. Potential
186 candidate genes could include those involved in the anthocyanin pathway, which influences red –
187 purple coloration in a number of plants (Asen et al. 1972, de Pascual-Teresa et al. 2002, Abdel-
188 Aal et al. 2006, Knievel et al. 2009). Further work will begin to elucidate the specific mechanism
189 influencing these color difference in invasive *G. paniculata* populations, as well as to explore
190 whether this color difference drives functional differences between the morphs.

191 In terms of management strategies, the purple and green-yellow morph are not distinct
192 species and are likely derived from the same genetic strain, so there does not appear to be a need
193 to manage these two morphs differently. One concern with the green-yellow morph initially
194 noted by TNC removal crews was that the taproot tended to be more diffuse than the purple

195 morph potentially making manual removal of these plants less effective. We are currently
196 examining root growth between the green-yellow and purple morphs under controlled conditions,
197 however, our lab's personal observations in the field have not found any indication that large
198 differences in root structure occur between mature plants of the two color morphs. Therefore,
199 current management approaches for these populations should be maintained to control the further
200 spread of *G. paniculata* throughout the Michigan coastal dune system.

201 **Acknowledgements**

202 This project was funded through an EPA-Great Lakes Restoration Initiative Grant and through
203 an NSF-REU grant. We would like to thank Shaun Howard from The Nature Conservancy for
204 his help in identifying the green-yellow morph and Benjamin Giffin for his help with sequencing
205 analysis. We would also like to thank Kurt Thompson and Brayden Partridge for assisting with
206 the figure construction.

207
208 **References**
209

210 Abdel-Aal, E.S.M., Young, J.C., and Rabalski, I. 2006. Anthocyanin composition in black, blue,
211 pink, purple, and red cereal grains. J. Agric. Food Chem. **54**(13): 4696-4704.
212
213 Asen, S., Stewart, R.N., and Norris, K.H. 1972. Co-pigmentation of anthocyanins in plant
214 tissues and its effect on color. Phytochemistry **11**(3): 1139-1144.
215
216 Bevill, R.L., Louda, S.M., and Stanforth, L M. 1999. Protection from Natural Enemies in
217 managing rare plant species. Conserv. Biol. **13**(6): 1323-1331.

218 Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., Zhu, Y., Ma, X., Gao, T., Pang, X., Luo, K., Li, X.,
219 Jai, X., Lin, Y., and Leon, C. 2010. Validation of the ITS2 region as a novel DNA
220 barcode for identifying medicinal plant species. PloS One **5**(1): e8613. doi:
221 10.1371/journal.pone.0008613L

222 de Pascual-Teresa, S., Santos-Buelga, C., and Rivas-Gonzalo, J.C. 2002. LC–MS analysis of
223 anthocyanins from purple corn cob. J. Sci. Food Agric. **82**(9): 1003-1006.
224

Darwent, A.L., and Coupland, R.T. 1966. Life history of *Gypsophila paniculata*. Weeds **14**(4): 313-318.

Department of Natural Resources (DNR). 2015. Michigan Invasive Species Grant Program Handbook. http://www.michigan.gov/documents/dnr/2015-MISGP-hndbk_491809_7.pdf?source=govdelivery

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32**(5): 1792-1797.

Emery, S.M., Doran, P.J., Legge, J.T., Kleitch, M., and Howard, S. 2013. Aboveground and belowground impacts following removal of the invasive species baby's breath (*Gypsophila paniculata*) on Lake Michigan sand dunes. Restoration Ecol. **21**(4): 506-514.

Fior, S., Karis, P. O., Casazza, G., Minuto, L., and Sala, F. 2006. Molecular phylogeny of the Caryophyllaceae (Caryophyllales) inferred from chloroplast matK and nuclear rDNA ITS sequences. Am. J. Bot. **93**(3): 399-411.

Jukes, T.H. and Cantor, C.R. 1969. Evolution of protein molecules. *In* Mammalian Protein Metabolism. Edited by H. N. Munro. Academic Press, New York. pp. 21-132

Karamanski, T.J. 2000. Nationalized Lakeshore: The Creation and Administration of Sleeping Bear Dunes National Lakeshore. Online Book. National Park Service, Department of the Interior. http://www.cr.nps.gov/history/online_books/slbe/

Kumar, S., Stecher, G., & Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33**(7): 1870-1874.

Lee, S. C., Wang, C. H., Yen, C. E., and Chang, C. 2017. DNA barcode and identification of the varieties and provenances of Taiwan's domestic and imported made teas using ribosomal internal transcribed spacer 2 sequences. J. Food Drug Anal. **25**(2): 260-274. doi: 10.1016/j.jfda.2016.06.008

Leege, L. M., Murphy, P. G. 2001. Ecological effects of the non-native *Pinus nigra* on sand dune communities. Can. J. Bot. **79**(4): 429-437.

Stevens, O. A. 1957. Weights of seeds and numbers per plant. Weeds **5**: 46-55.

Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol. Biol. Evol. **9**(4): 678-687.

The Nature Conservancy (TNC). 2014. Lake Michigan Coastal Dunes Restoration Project. 2013 Field Season Report. The Nature Conservancy and Partners, Lansing, Michigan

Figure Legends:

Figure 1: (A) Green-yellow morph, (B) common purple morph and (C) Stem of the green-yellow and purple baby's breath morph found in Sleeping Bear Dunes National Lakeshore.

Figure 2: Phylogenetic construction of the purple and green-yellow baby's breath color morphs in relationship to *G. paniculata* and *G. elegans* reference sequences. All evolutionary histories were inferred using maximum likelihood methods. (A) Phylogeny based on RbcL, MatK, and ITS2 combined, (B) phylogeny based on the RbcL region, (C) phylogeny based on the MatK region, (D) phylogeny based on the ITS2 region. For RbcL and ITS2 we used a Jukes Cantor model of molecular evolution (Jukes and Cantor, 1969). For MatK and the combined data set we used a Tamura 3-parameter model of molecular evolution (Tamura 1992).

Figure 1

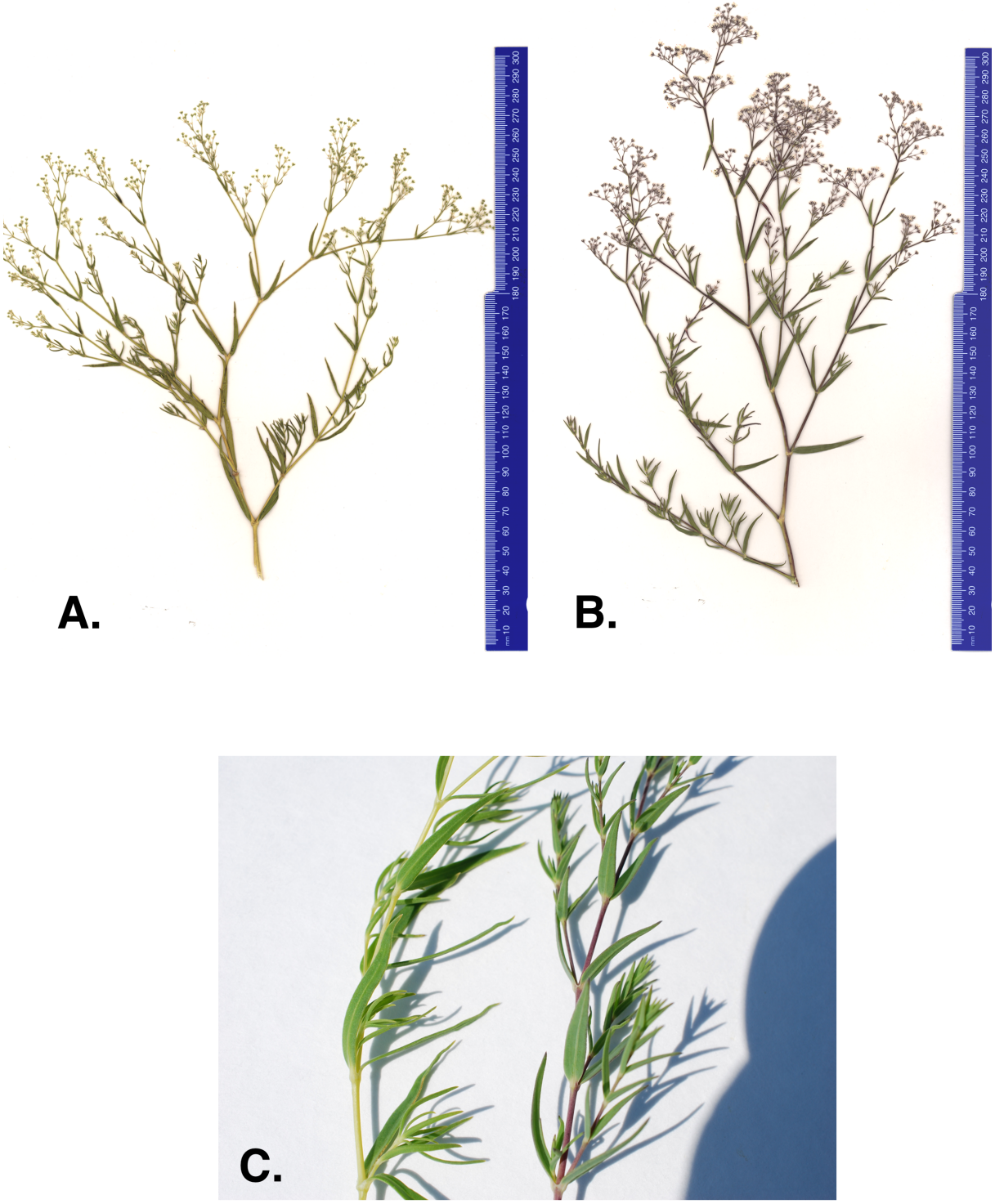
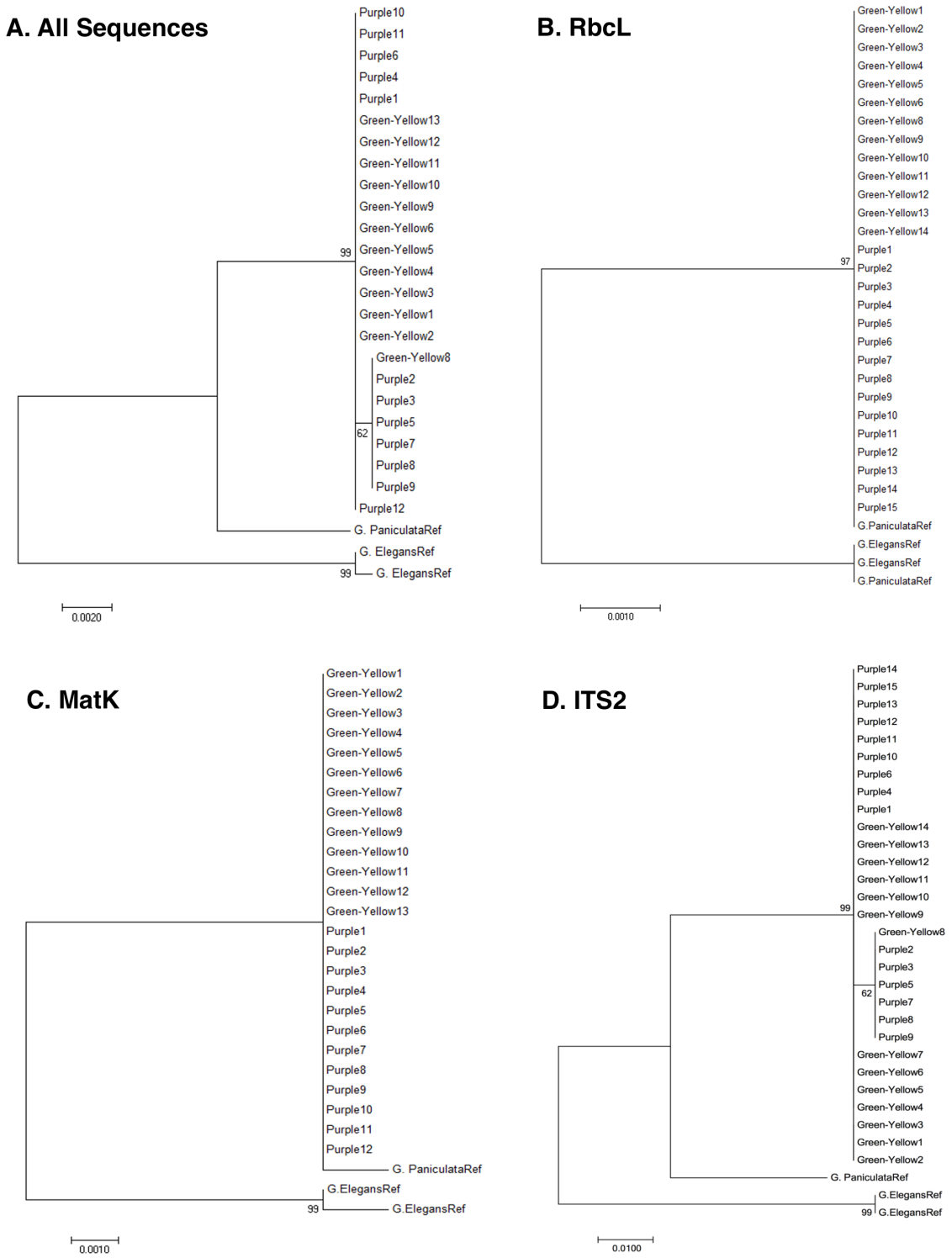


Figure 2


A. All Sequences
Purple10
Purple11
Purple6
Purple4
Purple1
Green-Yellow13
Green-Yellow12
Green-Yellow11
Green-Yellow10
Green-Yellow9
Green-Yellow6
99
Green-Yellow5
Green-Yellow4
Green-Yellow3
Green-Yellow1
Green-Yellow2
Green-Yellow8
Purple2
Purple3
Purple5
62
Purple7
Purple8
Purple9
Purple12
G. PaniculataRef
G. ElegansRef
99
G. ElegansRef
0.0020
B. RbcL
Green-Yellow1
Green-Yellow2
Green-Yellow3
Green-Yellow4
Green-Yellow5
Green-Yellow6
Green-Yellow8
Green-Yellow9
Green-Yellow10
Green-Yellow11
Green-Yellow12
Green-Yellow13
Green-Yellow14
Purple1
97
Purple2
Purple3
Purple4
Purple5
Purple6
Purple7
Purple8
Purple9
Purple10
Purple11
Purple12
Purple13
Purple14
Purple15
G.PaniculataRef
G.ElegansRef
G.ElegansRef
G.PaniculataRef
0.0010
C. MatK
Green-Yellow1
Green-Yellow2
Green-Yellow3
Green-Yellow4
Green-Yellow5
Green-Yellow6
Green-Yellow7
Green-Yellow8
Green-Yellow9
Green-Yellow10
Green-Yellow11
Green-Yellow12
Green-Yellow13
Purple1
Purple2
Purple3
Purple4
Purple5
Purple6
Purple7
Purple8
Purple9
Purple10
Purple11
Purple12
G. PaniculataRef
G.ElegansRef
99
G.ElegansRef
0.0010
D. ITS2
Purple14
Purple15
Purple13
Purple12
Purple11
Purple10
Purple6
Purple4
Purple1
Green-Yellow14
Green-Yellow13
Green-Yellow12
Green-Yellow11
Green-Yellow10
99
Green-Yellow9
Green-Yellow8
Purple2
Purple3
Purple5
62
Purple7
Purple8
Purple9
Green-Yellow7
Green-Yellow6
Green-Yellow5
Green-Yellow4
Green-Yellow3
Green-Yellow1
Green-Yellow2
G. PaniculataRef
G.ElegansRef
99
G.ElegansRef
0.0100